THE PERMEABILITY OF INSECT CUTICLE

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THE PERMEABILITY OF INSECT CUTICLE

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ABSTRACT

The penetration of malathion through the intact cuticle of American cockroaches, Periplaneta americana L. has been studied. Prior to studies on malathion, the effect of water on permeability of the cuticle was investigated. It was found that water immersion or high humidity treatment changes the nature of the cuticle. The minimum individual variation in the rate of penetration of radioactive malathion was obtained by using dead, intact P. americana.

The rate of entry of malathion was found to deviate from the theoretical rate of diffusion. The deviation was due to adsorption of malathion into the cuticle: a large amount of malathion was discovered in the cuticle even immediately after its application on the cuticle. It was found that, if the adsorption of malathion was removed, the rate of penetration of malathion through the cuticle of roaches was at the theoretical rate by diffusion, and that the adsorption could be explained by means of Langmuir's adsorption equation.

The mode of entry of malathion into the insect body was also investigated by using radioautography: malathion was found to enter mainly through the legs and antennae, and not through the spiracles as once supposed. Malathion is rapidly adsorbed by the cuticle for the first hour and gradually penetrates into the insect body. The adsorbed malathion can be recovered by extracting the cuticle with hot water, but not with wax solvents. Thus it is reasonable to assume that adsorption takes place at the hot water soluble layer of the cuticle which consists of protein.

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REVIEW OF LITERATURE.

1.1. The Basic Problems.

The insect cuticle has been studied from the view point of the insects' ability to resist desiccation. If a drop of water, which is about the average size of an insect, is left on a table, the drop will evaporate in a few minutes. The cuticle is characterized not only by its great resistance to the loss of water by evaporation, but also by its power of adsorbing water. Pal (1947) observed that drops of water placed on a leg of a cockroach make their appearance in the trachea below. Also Lees (1946, 1947) has proven that many species of ticks can take up water from the air even when it is far below saturation, and intake takes place through the cuticle. This is very surprising. It is not difficult to find a membrance which can adsorb water along the concentration gradient of water, but is almost impossible to find a membrane which can adsorb water against the water concentration.

Another characteristic of the cuticle is its high susceptibility to the entry of insecticides. Regardless of its hard appearance, the cuticle is not resistant to the penetration of insecticides. The difference between the LD-50 in topical application and oral application or in-

jection is usually very little in insects. In other words, insecticides penetrate through the cuticle as if there were no barrier at all. On the other hand, in mammals the contact toxicity is much lower than that of oral or injection (Dresden & Krijgsman, 1948). This means that regardless of the hard appearance, the cuticle is a very poor protective membrane with respect to insecticidal attack and a very effective membrane with respect to water regulation.

Much work has been done in order to explain those peculiar characteristics of the insect cuticle in terms of its composition, chemical properties, physical nature and so on. Nevertheless, practically none of the workers could visualize the true picture of cuticular penetration. The main difficulty in this problem appears to be that they have been trying to reduce the problem to the mechanical composition of the cuticle only and have not considered the problem from the physiological point of view. In fact, the dead cuticle is very different from the living one. For instance, the asymmetrical nature of the cuticle as exhibited by the ratio of inward to outward penetration in the isolated cuticle has been proven to be 2:1, at the very most 5:1 (Beament, 1954), rather than 100:1 as had been reported previously. In living insects the ratio is assumed to be much higher. Moreover the

phenomenon of water intake against the gradient of water concentration has never been observed in the cuticle from a dead insect.

1.2 Permeability of the Cuticle to Water.

1.2.1. Cuticle hardening and permeability.

It has been assumed that the tanning process with the subsequent condensation of protein can induce a certain degree of impermeability. It is natural to assume that the hard, dark cuticle is more resistant to the loss of water than the white, soft cuticle. In fact, Kalmus (1941 a.b.) found that dark mutants of Drosophila survived longer in a dry atmosphere than light mutants. Many workers who have studied the correlation between tanning and permeability failed to study the problem with respect to the moulting process. Wigglesworth (1945) mentioned that the fully hardened puparium of Calliphora of from 24 to 48 hours old is highly water-proof, but if the most superficial layer is abraded by rubbing it lightly with alumina dust it loses this property and quickly dries up. This does not happen in the 3 days old puparium: however this is not because the condensed chitin protein complex in the puparial shell is becoming impermeable, but because the water-proof cuticle of the next stage has been formed inside. Beside this there are certain dangers in confusing the physiological changes occurring during the moult with purely mechanical changes in the nature of the cuticle.

1.2.2. Permeability and the structure of the cuticle.

In 1944 Wigglesworth studied the drying up of insects when they moved in contact with certain fine dusts. The phenomenon was proven to be due to the abrasion of the true water-proofing layer. The site abraded can be made visible by immersing the living insect in a solution of ammoniacal silver hydroxide (Wigglesworth, 1944). Although it has been found that the effect of the hard abrasion is not only the removal of the wax layer, but also the damaging of many different layers (Richards, 1957), this gave a useful suggestion for the study of the water-proofing mechanism, particularly noting the role of the wax layer. The significance of the wax layer of the insect cuticle in the water-proofing mechanism has been found by removing it with wax solvents (Wigglesworth, 1945; Beament, 1945). It was observed that the evaporation of water showed a sudden increase under these conditions.

In 1941 Hurst made an important step showing that the isolated insect cuticle is itself asymmetrical: water has been found to pass through the isolated cuticle faster inward than outward. Although he made an experimental error using a water immersed cuticle, as Beament (1948) pointed out, the result introduced the idea of studying what kinds of mechanisms are responsible for the asymmetrical behavior

of the insect cuticle. He claimed that the rate of passage of water from endocuticle to epicuticle was just as one hundred times as that of the reverse. On the basis of this observation, he suggested that the insect epicuticular wax layer is composed of fatty acids in a lamellar structure; perpendicularly arranged lipoid layers can regulate the water penetration in either direction. Many questions have arisen concerning this hypothesis. Wigglesworth (1948) pointed out the following: (1) how can the structure be explained from the view point of cuticle formation? (2) the linkage between lipids and protein is not clear, (3) the lamellar structure of cuticle found by Fraenkel and Rudall (1948), quoted by Hurst, is only true in endocuticle, (4) the epicuticle cannot be considered as a membrane which is spatially separated from the liquid water phase by the endocuticle as Hurst (1948) remarked. On the other hand, the monolipoid layer theory of Wigglesworth and his co-workers does not perfectly explain the phenomenon: there is no evidence that a lipoid layer adsorbed onto a solid structure can prevent water evaporation, and selectively take in water as Schulman (1948) noted. It has been confirmed by x-ray diffraction that a collodion membrane is not soluble in water, but swells so that there is an increase in the effective free

pore space within the membrane. The results were compared with the known crystalline structures of long-chain fatty acids, paraffins, and the free and bound lipids in the epicuticle of Calliphora pupal exuviae (Hurst, 1950). In this experiment it has been proven that artificial collodion membranes give electron diffraction patterns similar to those of the insect cuticle, indicating that the free, or thermolabile waxes in the epicuticle consist of a three dimensional arrangement of orthorhombic micro-crystals oriented perpendicular to the cuticle surface. Although the work has contributed to the study of penetration through the cuticle, the perfect explanation cannot be merely this, as Wigglesworth pointed out. As mentioned before, this asymmetrical phenomenon in the living cuticle is very well demonstrated.

1.2.3. The nature of the cuticular wax.

The nature of the wax of the epicuticle has been studied by many workers, since Beament (1945) and Wigglesworth (1945) pointed out its role in evaporation with special reference to the melting point. In Pieris brassicae larvae there is an inverse relationship between the humidity in which the insect was reared and the amount and melting point of the cuticular wax (Koidsumi, 1953). Also, it has been noted

that the moth Dictyoploca japonica B. yields a larger amount of epicuticular lipids with higher melting points when it is reared at high temperatures (Koidsumi, 1952; Miyazaki, 1952). It is striking indeed that the melting point of the cuticular waxes can be changed by the circumstances, for if it is entirely true, the water controlling mechanism of the insects might not be merely mechanical adaptation. Also, Beament (1951) reported that the grease freshly extracted from the cuticle of Periplaneta has a melting point of about 34°C., when exposed to the air for some months, it gradually hardens to a wax melting at about 55° C. This change does not occur if the grease is sealed in a tube, even when exposed to oxygen in the light; on the other hand, hardening can be brought about within one hour if the grease is heated in air or in vacuo at 100°C. The wax extracted from the pupa of Tenebrio, examined by transmission electron diffraction, gives a ring pattern typical of an orthorhombic straight chain with spacing a: 7.36Å, b:4.89 Å, but this space is not changed at all by high temperatures until complete fusion of the wax had occurred (Holdgate and Seal, 1956). This striking finding led these authors to reinvestigate the nature of the critical temperature, which was named for the point of temperature where the transpiration of water showed a sudden

increase. According to Holdgate and Seal, the general equation for diffusion of a fluid through a sheet of solid is

$$VC_g = D(C_1-C_2)t/L \qquad (1)$$

where C_g is the concentration of fluid which has diffused through the sheet into a volume V after a time t. D is the diffusion constant, and L is the thickness of the sheet. C_1 and C_2 and concentrations of the fluid in each side of the membrane. For a gas this can be written in terms of pressure.

$$V_p/t = D(P_1-P_2)/L \qquad (2)$$

Assuming the gas is ideal, the equation can be rewritten as

$$m = Dt(P_1-P_2)/LRT \qquad (3)$$

where m is the mass of water diffusing, R is the gas constant, and T is the absolute temperature. The difference of the vapor pressure of water inside and outside, P, is given by:

$$Ae^{-B/T} \qquad (4)$$

whence A and B are constants. Therefore

$$m = (DtA/LRT)\ e^{-B/T} \qquad (5)$$

The equation in unit time penetration can be written considering the constants

$$m = Ze^{-Y/T} \qquad (6)$$

where Z and Y are constant. If this relation held strictly for the transpiration of water through the insect cuticle, a straight line should be obtained when $\log_{10} m$ is plotted against 1/T. In permeable cuticles of aquatic insects or in Tenebrio larvae, in which the surface of the cuticle has been slightly abraded, this relation holds, and a straight line is obtained. On the other hand, in insects with a complete layer of water-proofing wax, such as Tenebrio pupae or Rhodnius, the points on the logarithmic plot depart significantly from linearity. In other words, the sudden change observed by Ramsay (1935), Wigglesworth (1945), and Beament (1945) in water evaporation was not due to the melting point of the wax of the cuticle. It was unfortunate that in the original curves of transpiration published by these authors, the rate of loss of water was plotted directly against the temperature, and the steeply rising drying power of the air was not taken into account. Since the coincidence of critical temperature and the melting point of the cuticular wax was supposed to be one of the biggest supporting

phenomenon for the water-proofing mechanism of the wax layer, the same authors suggest that the primary wax layer alone does not seem to be responsible for the water proofing of the cuticle; the cement layer may well play an important part in water proofing, besides acting as a protective sheet. Nevertheless, there is a proof that the wax layer is not always the main factor regulating the asymmetrical nature of the insects' cuticle, regardless of the fact that the wax layer has been studied by many workers as one of the basic problems: for instance, larvae of higher Diptera possess no wax layer, and yet they are highly resistant to desiccation (Richards & Fan. 1949). This fact was confirmed by Dennel (1946) and Wolf (1954). In this case, it has been found that the penetration of water (Wolf, 1955) and the property of asymmetrical penetration (Richards et al., 1953) are both controlled by the cuticulin layer of fly larvae.

1.3. Studies on the Permeability of the Cuticle to Materials other than Water.

The insect cuticle is very susceptible to the entry of insecticides. This phenomenon, however, has been neglected by many workers. Studies on the basic mechanism of penetration of compounds other than water are needed.

One of the most important standpoints of this field, which is different from water transpiration studies, can be pointed out: the water intake through the cuticle is achieved against the concentration of water, whereas intake of insecticides or any other compounds is performed along the concentration of the compounds. Studies on the penetration of insecticides or other compounds should be carried out bearing this in mind.

It has been said that the cement and wax layers which restrict the cuticular penetration of water also oppose the entry of insecticides; their entry is enormously accelerated after these layers have been injured by abrasion (Wigglesworth, 1945). It seems also generally true that the presence of wax solvents will facilitate the passage of toxic substances into insects (O'Kane _et al._, 1940; Morozov, 1935).

The entry of alcohol through the cuticle of Calliphora larvae is accelerated in the presence of kerosene, this effect being attributed to the increased permeability of the outer lipid layer in the presence of polar substances (Hurst, 1940, 1943). Pyrethrum enters the cuticle of Rhodnius progressively more rapidly when dissolved in the more volatile paraffins. In this case the thickness of the endocuticle is important in slowing down the rate of entry (Wigglesworth, 1942a). In this study it was also noted that oils , particularly in the presence of some oleic acid, will pass through the cuticle, and appear in the cells of the general epidermis and in the dermal glands below. Wigglesworth has mentioned that oils can never be detected in the substance of the cuticle, presumably they pass through in droplets beyond the resolving power of the microscope. On the other hand, it has been shown that certain organic solvents of diphenylamine, such as the cresols, benzyl alcohol and 4-methyl-cyclohexanol greatly increase the rate of action of insecticides (Webb and Green, 1946). These authors have found that not all solvents of beeswax, however, increase the rate of diffusion of the insecticides across the cuticle; only those which possess a high partition coefficient between beeswax and water.

They have also explained that the main function of those solvents is to pass readily from the wax phase into the aqueous phase and the concentration of insecticides in the presence of the wax phase will then tend to increase and this will favor its rapid diffusion across the interface. Although much information in terms of the biological and toxicological aspects of insecticide penetration can be learned from these preliminary papers, they can only be regarded as qualitative in nature.

In 1949 Richards and Fan made an important step in giving the first quantitative data. These authors were concerned with variations in terms of permeability of the larval cuticle of Phormia regina with regard to KCL, methylene blue, and $CoCl_2$; variations of 3-4X the lowest value were usual within a single lot of larvae, and extremes of 8.7X were obtained for KCL, 10X for methylene blue, and 18X for $CoCl_2$. Approximately twice this amount of variation is found between values for cuticles from individuals of different cultures of only approximately the same age and history. The variation of permeability has been also pointed out by Ricks and Hoskins (1948). On the other hand, fairly constant values were obtained by Treherne (1957) with regard to the diffusion of non-electrolytes through the isolated cuticle of Schistocerca gregaria.

Treherne (1957) has studied the ability of non-electrolytes to diffuse in connection with the solubility of the penetrating molecules in water, in the whole cuticle, and in the wax layer alone. It has been found that the log. of the permeability constant varies proportionally to the long. of solubility of the penetrating molecules to water. The per cent standard error((S.E./Mean) X 100) in this experiment is calculated to be approximately 20%.

2. THE BASIC NATURE OF THE CUTICLE WITH REGARD TO WATER PERMEATION.

2.1. Introduction.

It seems that the wax layer alone cannot possess the asymmetrical nature of the cuticle, since it has been shown that some dipterous larvae have no wax layer, and yet they are highly resistant to desiccation; the factor which causes the asymmetrical nature of the insect cuticle must be common throughout all insects. The main difficulty in this problem is that a perfect cuticular sheet can never be obtained. For instance, if the cuticle is dissected in water the water soluble part of the cuticle is dissolved, and then the cuticular composition is obliged to change. Particularly this is true in P. americana; in this animal it is well known that a drop of water deposited on the surface of the cuticle is almost immediately covered by a grease film (Ramsay, 1935). Also Holdgate (1955) proved that if the cuticle was immersed prior to experiments, the contact angle of a water droplet decreases to yield a constant value.

It has been pointed out by Beament (1954) that a very high asymmetry value obtained by Hurst (1941) is due to the fact that Hurst used a cuticle sheet which had been preserved in water. As far as our present knowledge is concerned, data in this field are still rather qualitative in nature. For instance, in most cases humidity as an experimental condition is not recorded. This is serious, since penetration of water decreases proportionally as humidity increases at least as far as the theory of natural diffusion is concerned.

It must be mentioned that it is not the main object of this part of the experiment to investigate the reason of the asymmetry of the insect cuticle to water permeation. Here experiments were undertaken in order to find the real nature of the cuticle with respect to treating methods, experimental conditions such as humidity or temperature, variation of data, meaning of the isolated cuticle, the intact, the alive insects' cuticle etc., so that the study of the penetration of non-electrolytes can be carried out as precisely as possible.

2.2. Methods and Material.

2.2.1. The nature of the material.

American cockroaches, Periplaneta americana L., were used for all experiments. They were reared on dog food and water at 22-27° C., and approximately 60% R.H. They were kept in a container 21 x 19 x 34 cm. in size; approximately 30 adults were reared in a pot. The whole body was used for the intact cuticle tests. To obtain membranes the abdomens of adults of P. americana were cut off and split longitudinally. The sheet was separated into two pieces: the dorsal and ventral sheets, and the attached tissue, mainly fat body was removed by gentle rubbing with a cotton swab. In this case the use of water was avoided in order to differentiate the washed and wet cuticle.

The epicuticle of P. americana consist of four layers as Dennell and Malek (1954) have described; an innermost cuticulin layer, a very thin paraffin-containing layer, a wax and an outermost cement layer. They are very susceptible to abrasion and easily damaged as Wigglesworth has mentioned (1945). The cuticle, therefore, was handled very carefully.

2.2.2. Water permeation through the living and dead, intact and isolated cuticle.

In all cases the amount permeated was measured in terms of the weight loss of the original insect (Table 1), or that of tubes to which the isolated cuticle was attached (Table 2). The materials were kept in desiccators which were kept at various constant humidities by conc. H_2SO_4. The spiracles were carefully plugged with beeswax in order to eliminate the effect of transpiration through these organs. In each experiment the temperature was recorded, but recalculation of the value as to temperature was not done, since under the experimental conditions the error caused by the temperature is lower than that of the variation of each individual.

2.2.2.1. The living and dead, intact cuticle test.

The roaches were starved for three days in a desiccator chamber which had been kept at 40% R.H. and at 22.0 ± 0.5°C. Four female roaches were used and two of them were killed by HCN gas in a bottle. Wings had been removed before the experiment was started. Then the roaches were transferred into a desiccator which had been kept at 3.2% R.H., and the subsequent decrease in weight was recorded with a balance.

2.2.2.2. The isolated cuticle test (Table 2).

The cuticle isolated from a cockroach was attached by water glass with two iron washer rings to the bottom of a 5 mm. inside

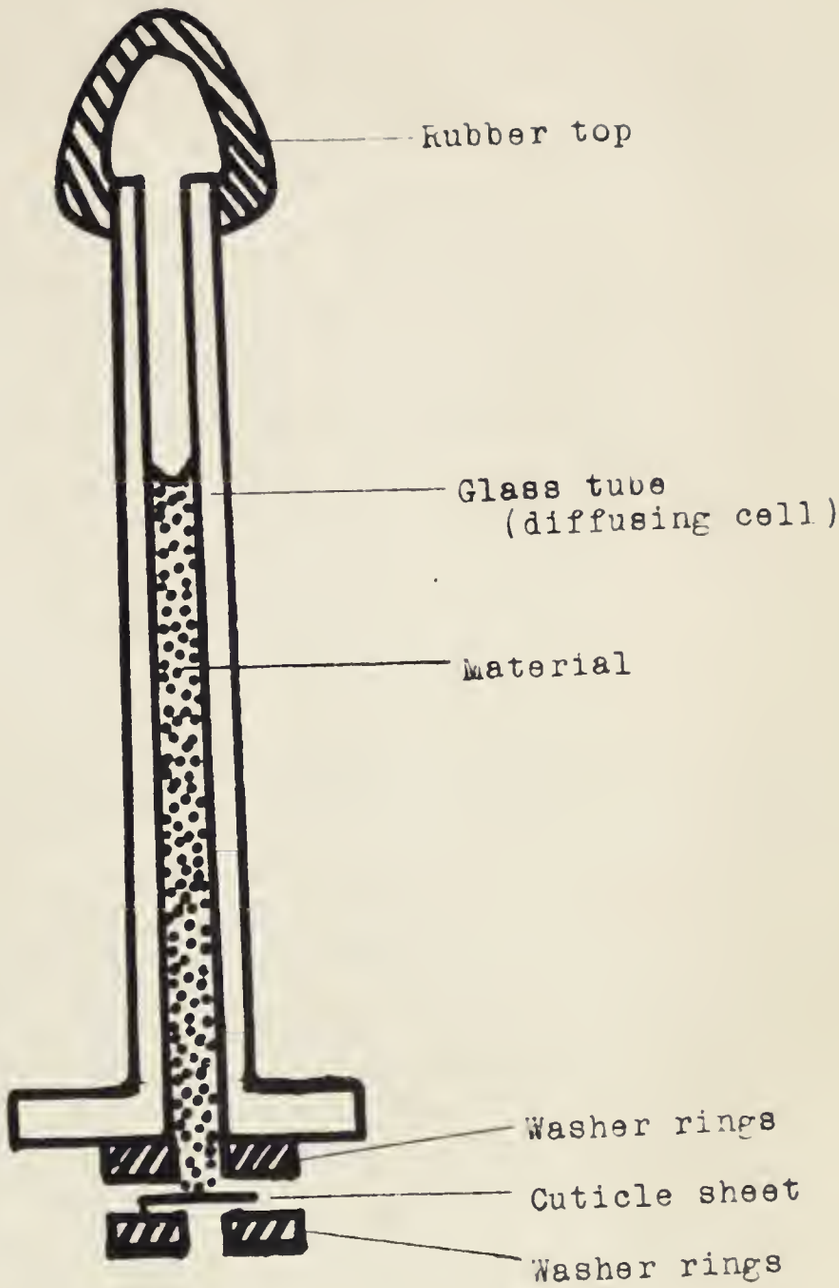


Fig. The diagrammatic vertical section of the apparatus used to measure the diffusion through the isolated cuticle.

diameter glass tube, of which the top was covered by a rubber cap to prevent evaporation of water from the tube (see Fig. 1.). The cuticle sheet was obtained from male roaches which had been kept 3 days in a desiccator chamber at 40% R.H. at 22 - 26' C., and dissected without using water as described before. After the cuticle was set on the tube, it was kept in a desiccator at 40% R.H. for three hours, then was transferred into another desiccator at 3.2% R.H. The tube was filled with 1 ml. of water which filled the lower one-third of the tube. The experiment was carried out at 22.5 ± 1'C.

2.2.3. The role of the wax layer; chloroform extraction procedure.

The intact cuticle was used for this experiment. The insects were killed by HCN gas described before. After the wings were removed the insects were immersed in 100 ml. of cold chloroform in a 500 ml. beaker. The insect was lifted by the legs with a pair of forceps after a given interval, and chloroform which was left on the surface of the cuticle, was removed with the use of a filter paper. After immersion the insect was kept at 3.2% R.H., and weight loss by evaporation of water through the cuticle was recorded.

2.2.4. The effect of water treatments on the nature of the cuticle.

2.2.4.1. The effect of pre-immersion (Table 4.).

Both living and dead insects were used for this experiment. The latter were killed by HCN gas. The water immersion followed. The insect was held in water by a pair of forceps by the wings. The capacity of the container of water was about 10 liters, and water was allowed to run from the bottom, and the wasted water was allowed to overflow (Holdgate, 1955). Throughout the experiment female roaches were used. After washing, the wings of the insects were removed and all roaches were placed in a desiccator which had been kept at 3.2% R.H. The experiment was carried out at 21.5 ± 0.5°C.

2.2.4.2. The effect of humidity on permeation.

The isolated cuticle was used for this experiment (Fig. 4). Four males were dissected to obtain the isolated cuticle sheet. The cuticle sheet from the ventral abdomen, was then attached to the measuring tube (Fig. 1). To these tubes 2 ml. of water were added, and rubber tops were set. The tubes were then kept in the desiccators which were kept at 100;80.5;37.1;18.8;3.2 % R.H. respectively by various concentrations of sulfuric acid. The changing of the weight was measured by a balance.

2.2.4.3. The wetting effect on water evaporation.

The effect of water on the cuticle can be divided into two factors: washing out effect and wetting effect. To study the wetting effect alone, the water evaporation after exposing the cuticle in high humidity, was measured in two females (Table 5). These two were exposed in 100% R.H. for 2 hours and one was killed by HCN gas. One of the controls which had not been exposed in the high humidity was killed by the same method. Then the insects were kept in a desiccator at 3.2% R.H. at 21.0 to 22.0°C., and the change of the weight of the insects was recorded.

2.2.5. The effect of temperature (Fig. 5).

The roaches which were used in this experiment had been kept in a clean jar without food for one week. One group of roaches was washed with distilled water for three minutes, and another was treated with acetone as well. The rest were kept as controls. They were killed before the treatment. Each group was kept separately in a 50 ml. Erlenmeyer flask and placed in a desiccator at 60% R.H. The desiccator was set in a water bath container with 10 liters of hot water and temperature was regulated by a thermostat.

The whole set was allowed to stand for one hour at the given temperature and the weight decreases by evaporation was measured. The experiment was conducted at 24, 34, 44, 54, and 64'C, using different batches of roaches for each temperature setting.

2.3. Results.

2.3.1. Water permeation through the living, dead intact and isolated cuticle.

The results are summarized in Table 1. In this type of permeation, it can be reasonably assumed that the amount of permeation is proportional to time within some limit. The highest correlation with linearity was observed in the dead intact cuticle, and lowest linearity was shown by the living insects. It is noteworthy that the evaporation of water of living insects deviate from the straight line about 25 hours after being set in an extremely low relative humidity atmosphere. The role of the spiracle in permeation of water in terms of evaporation was not as great as assumed before, but it showed a parallel correlation with the dead control. The relation can be seen in Fig. 2. Different individuals showed slightly different values, but the characteristic of the curve was the same in any case. It is very interesting to know the variation of the rate of evaporation in unit time. The % standard error was the highest value (0.20) in the living insects while that of the isolated cuticle was relatively low (0.06); the % standard error was the lowest in the intact cuticle (0.05). The fact is

rather surprising, since it was supposed that the isolated cuticle would yield a high constancy due to the set experimental conditions. In the isolated cuticle of which epicuticle was faced to the desiccator, it can be observed that the penetration decreased as time passed. It is suspected, therefore, that the water passage might be affecting the nature of the cuticle itself. Hence the effect of preliminary water treatment was subsequently reinvestigated.

Table 1. Water evaporation through living and dead intact cuticle of P. americana with time.

(Mg./hr./gm.initial wt.)

di- n of ect	Amount of time in desiccator (hours)											Mean evap. rate
	2	4	6	8	18	20	22	24	26	28	30	
ing n spi- les	0.82	0.82	0.12	1.45	1.09	0.94	1.25	0.51	0.23	0.04	0.43	0.70 ±0.14
d sed racle	0.49	0.34	0.37	0.86	0.86	0.41	0.78	0.56	0.49	0.89	0.37	0.58 ±0.06
d n spi- les	1.38	1.08	0.89	1.42	1.31	1.23	1.53	1.23	1.04	1.49	1.04	1.24 ±0.06

Temperature 22.5'C
females
average of four roaches in each time

Table 2. The effect of time on the rate of water evaporation through the isolated cuticle of *P. americana*.

(Mg. of water/hr/mm^2 of cuticle)

Direction of penetration	Amount of time in desiccator (hours)							Mean evap.
	5	10	15	20	25	30	35	
Epicuticle to Endocuticle	5.4	7.6	8.5	9.3	9.4	9.9	6.4	8.07 ±0.59
Endocuticle to Epicuticle	7.8	6.6	6.0	5.8	6.0	5.3	4.9	6.06 ± 0.36

Temperature 22.5 ± 1°C

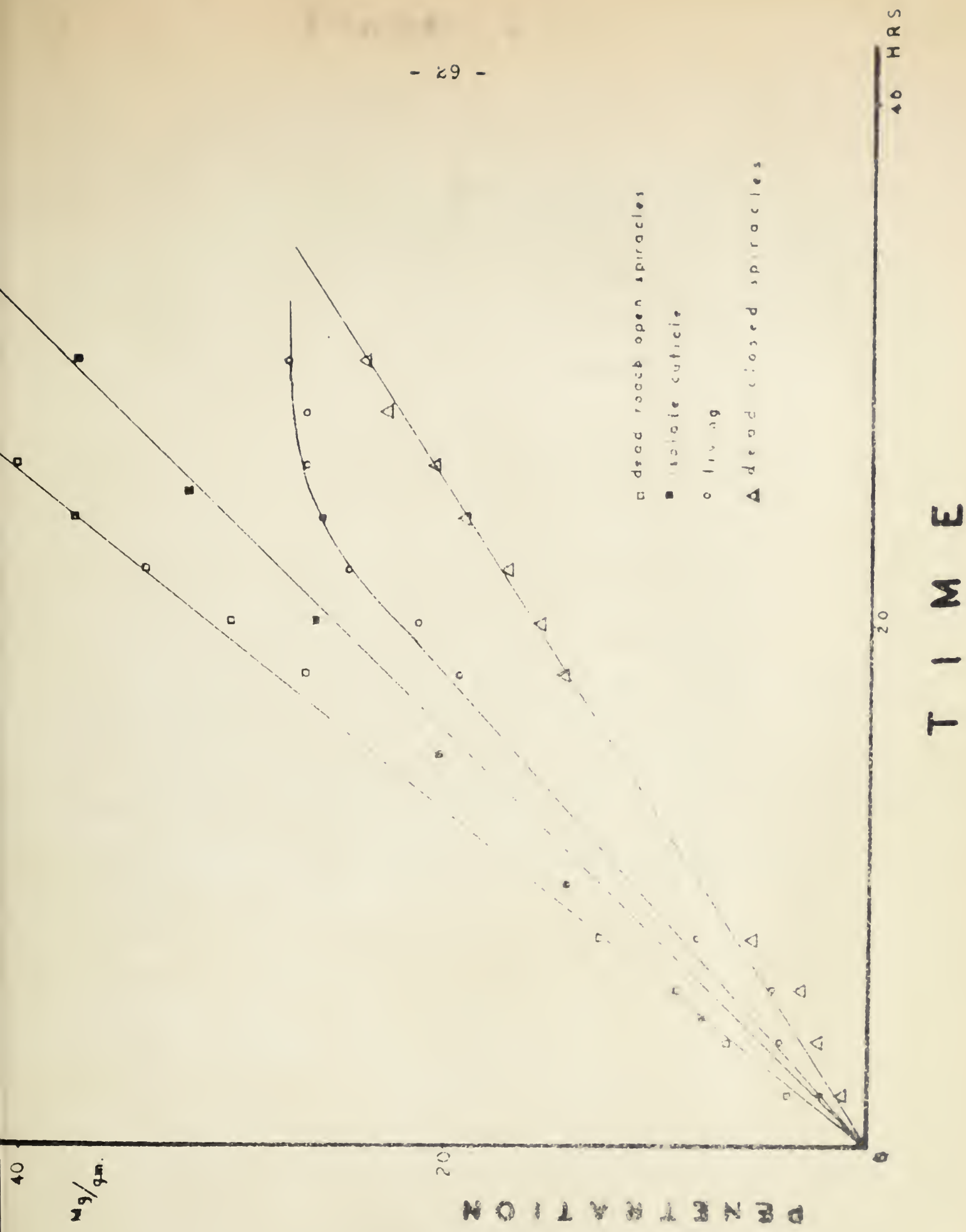


Fig. 2. The permeability to water of the living, dead and isolated cuticle.

2,3.2. The effect of chloroform extraction.

The results are presented in terms of chloroform extraction periods, and the rate of evaporation at a given time after the treatment (Table 3). No sudden increasing of evaporation was recorded as period of extraction became longer. That is, if the wax layer forms a distinct layer, there should be a time when all the wax layer is dissolved away, and when penetration increases suddenly. It seems that almost all of the wax is removed at the first moment, of chloroform extraction, but that the rest of the wax is tightly impregnated into the cuticle, and very difficult to be removed completely. After 30 min. extraction reaches its maximum (Fig. 3). Also it is remarkable that the rate of water evaporation decreases with time. For instance, in 10 sec. extraction, the amount of water evaporated per hour was 52.9 mg. at the first an hour, and 9.1 mg. per hour at 14 hours later (Table 3).

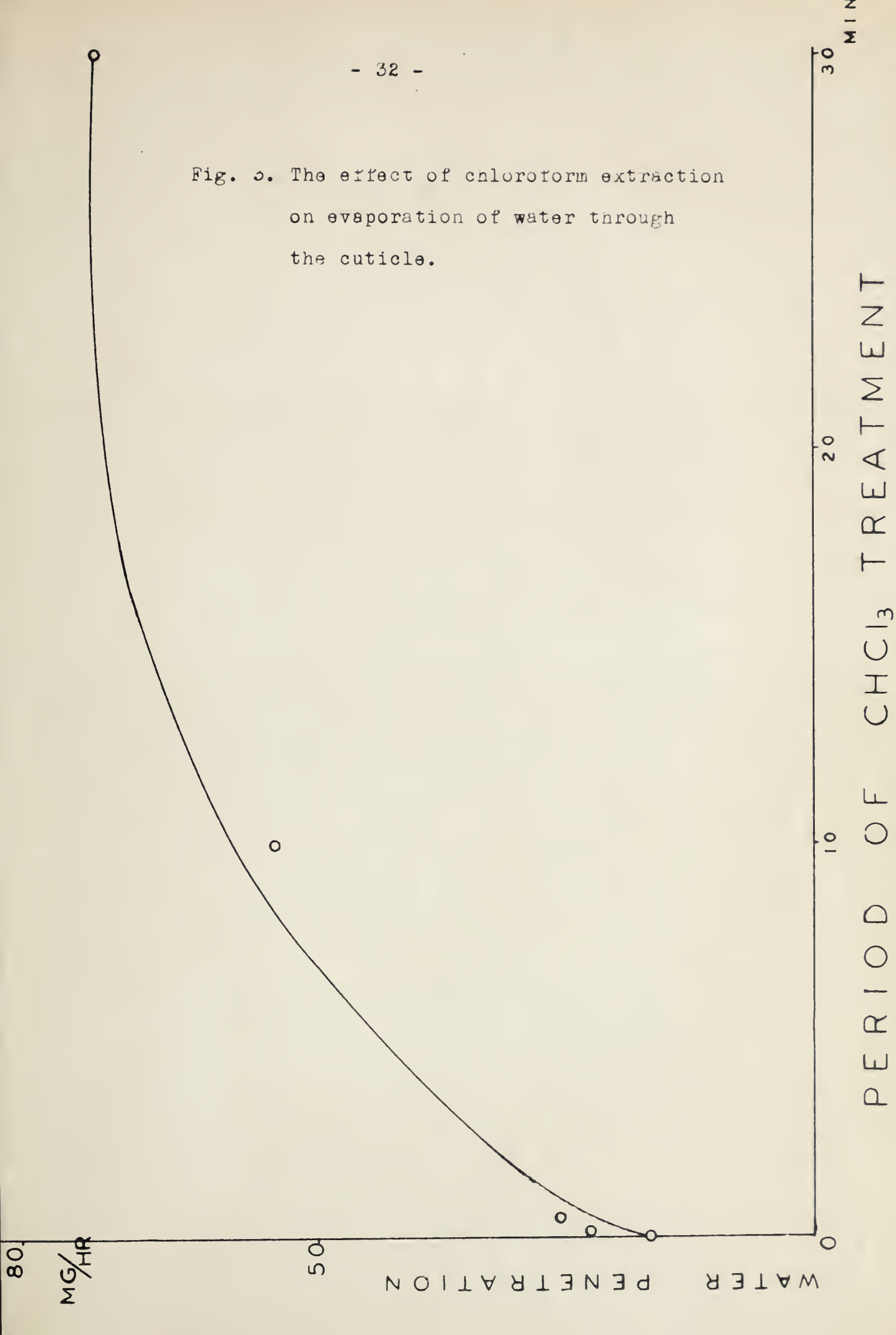
Table 3. The effect of chloroform extraction on water evaporation through the dead, intact cuticle.

(Mg./hr./body)

Time of extraction	Time after the extraction (hours)				
	1	2	3	13	14
10 sec.	52.9	22.7	20.9	10.1	9.1
30 sec.	42.0	25.6	23.5	10.5	9.2
10 min.	74.2	54.7	----	27.1	17.2
30 min.	77.5	72.0	----	35.5	18.1
0	22.8	16.7	16.5	----	----

Temperature 21.5 ± 1°C
3.2% R.H.

Fig. 5. The effect of chloroform extraction on evaporation of water through the cuticle.



2.3.3. The effect of water on the nature of the cuticle.

2.3.3.1. The effect of pre-immersion on permeation of water through the cuticle.

A higher evaporation rate was observed in the cuticle of dead roaches which was preliminarily washed with water as compared with untreated cuticle (Table 4). However, the rate of increase was the lowest in living insects, whereas the rate goes up to about 10 times in the case of the P. americana, which were treated with chloroform and water; the rate is very high as compared with the data which are obtained from the insects treated with chloroform alone. However, here again, the rate tends to decrease as time passes. The difference is insignificant. Also, it was found that the rate decrease continuously for about 9 hours and reaches a constant value: 1.2 to 1.6 X as compared with the control. The amount which is presented here can be regarded as due to the washing out effect of water, and a relatively high permeation rate at the first 9 hours or so is regarded as due to the wetting effect of water. An increase of permeation by washing out some substances from the cuticle is relatively low as compared with wetting effect of water on the cuticle.

Table 4. The effect of water immersion of the cuticle on the rate of water evaporation through the intact cuticle.

(Mg.of water/hr./body)

Condition of the insect	Treatment	Amount of time in the desiccator (hours)			
		1	2	5	6
Living	Control	----	3.18	----	----
	1 min. water	----	2.99	----	----
	5 min. water	----	3.44	----	----
Dead	Control	2.6	2.8	2.8	2.3
	1 min. water	6.7	----	3.7	3.1
	5 min. water	5.1	----	4.3	3.6
Dead	Control	1.6	2.4	----	----
	(1 min.water 1 min. chloroform	10.3	8.6	----	----
	(5 min.water 1 min. chloroform	10.7	9.9	----	----

Temperature 21.5 ± 0.5'C
3.2% R.H.

2.3.3.2. The wetting effect of water.

To investigate the wetting effect separately the insects were treated with high humidity atmosphere, as described before (2.2.4.2.), so that any kind of washing out effect would be avoided. The results are shown in Table 5.

It was found that high humidity pre-treatment increased evaporation from the dead, intact, roaches, but did not increase the rate of evaporation in living insects

Table 5. The effect of pre-treatment of the cuticle with high humidity on the rate of evaporation of water through the intact cuticle.

(Mg./hr./body)

Condition of the insect	Treatment	Amount of water evaporated	Note
Living	Control	15.46	
	100% R.H. for 2 hours	13.19	9 hours in the desiccator
Dead	Control	16.5	
	100% R.H. for 10 min.	27.6	7 hours in the desiccator

Temperature 21.5 - 0.5'C
3.2% R.H.

2.3.4. The effect of humidity and temperature on water permeation.

The effect of temperature had been misunderstood for a long time (see literature review). According to Holdgate and Seal (1956) the logarithm of transpiration rate must be proportional to 1/T. It is very interesting to see the nature of the cuticle in temperature change with particular reference to the water washed cuticle and acetone washed cuticle.

On the other hand, the effect of humidity also cannot be neglected, regardless of the fact that the description is missing in most of data published. According to Fick's law, the speed of diffusion is proportional to the potential difference of concentration of the substance:

$$\partial s/\partial t = DA \cdot \partial c/\partial x \qquad (7)$$

where s is the amount of penetrated substance, D is the diffusion constant, A is the area contacting, c is the concentration of the penetrating solution, and x is the distance of the cross section of the membrane. In other words, $\partial s/\partial x$ is the speed of diffusion across the membrane, and $\partial c/\partial x$ is the gradient of the water potential between the two sides of the membrane (Davson and Danielli, 1952).

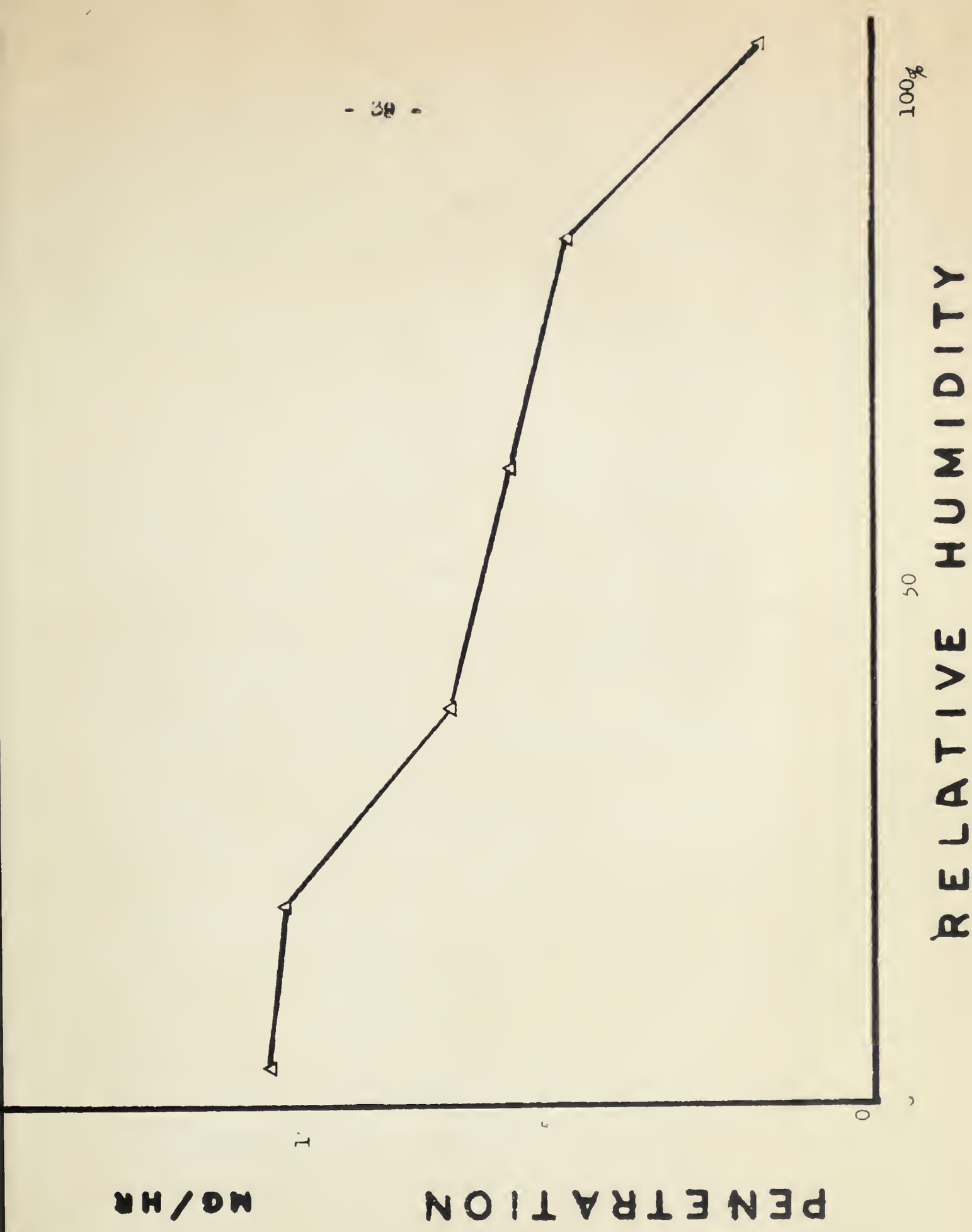


Fig. 4. The effect of humidity on the rate of penetration of water through the isolated cuticle.

Accordingly, the amount of transpiration through the cuticle must proportionally decrease as humidity increases.

2.3.4.1. The effect of humidity on the rate of evaporation.

The results have been shown in Fig. 4. As a whole it was found that the evaporation changes proportionally with humidity as assumed before. Some deviation from linearity, however, can be seen. The deviation might be due to the fluctuation of temperature during this experiment. Temperature must be strictly kept a constant in this kind of experiment.

2.3.4.2. The effect of temperature on the rate of evaporation.

The highest linearity was obtained in acetone washed insects, and the highest deviation was observed in dead ones. In addition, one can see the deviation very systematically: the more the line curves, the more penetration deviates from the theoretical rate of diffusion. The deviation of the result from the linearity means that in that membrane the natural diffusion is not taking place, but some functional, and mechanical force is preventing the membrane to allow natural diffusion.

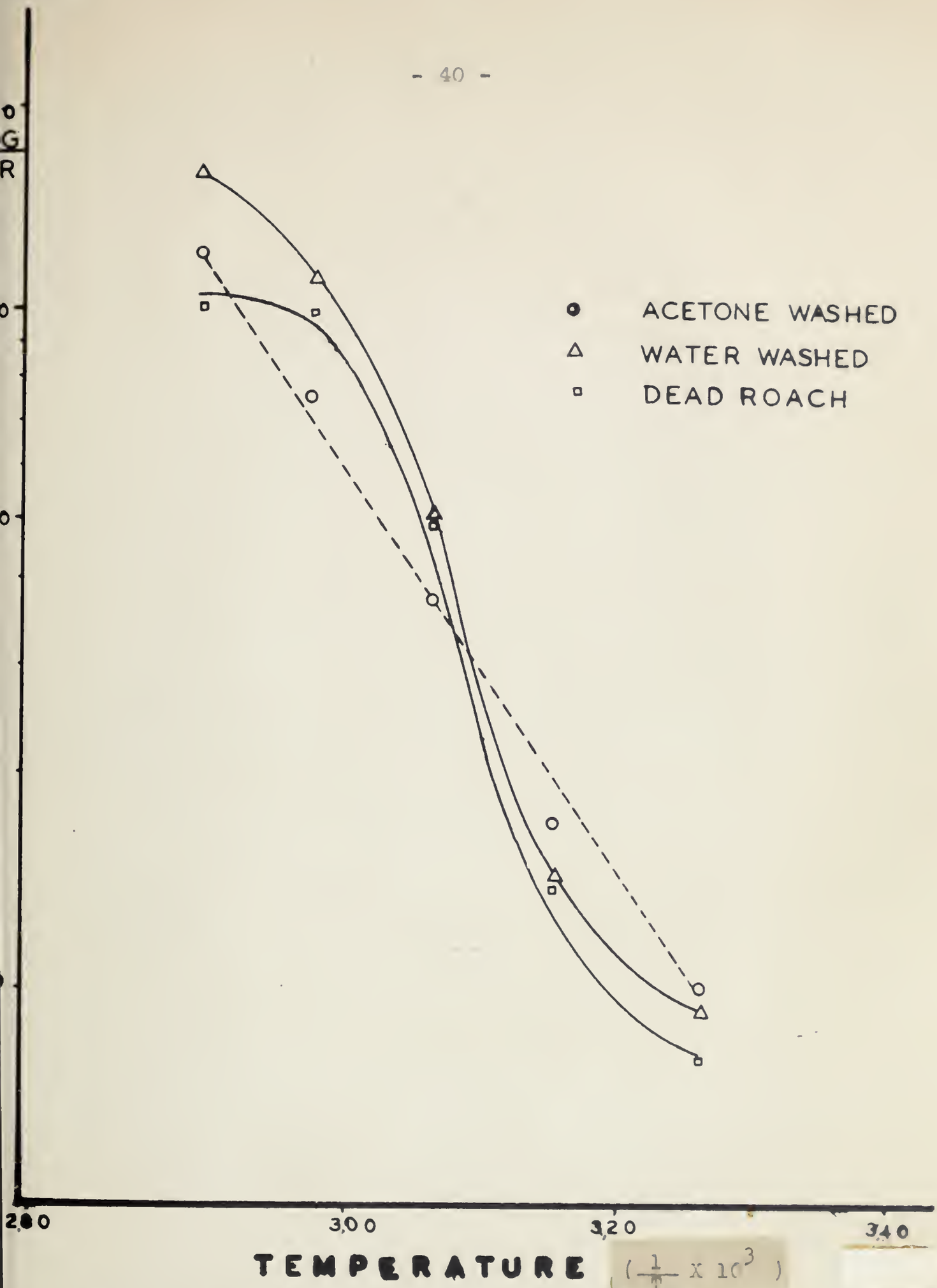


Fig. 5. The effect of temperature on the permeability of the cuticle to water.

2.4. Discussion.

In this experiment it has been shown that the wet cuticle has different properties from the dry cuticle, and that on the cuticle water premeation does not take place according to the natural diffusion theory. In other words the interaction between the penetrating substance and the membrane itself makes the problem more complicated. This interaction of the cuticle with water is greater in the isolated cuticle, and less in the living cuticle. The actual picture of the mechanism of cuticular penetration can be understood with respect to the difference between the living and the isolated cuticle, since this difference is due to the physiological function of the insect. The permeation rate is found to be about the same as in previous works published, but the value obtained in intact insects whose spiracles were closed was slightly less than the data of previous workers (e.g. Beament, 1945; Wigglesworth, 1945).

The isolated cuticle showed a relatively high variation despite the controlled experimental conditions.

There seems to be a danger in using abdominal cuticle as a typical cuticle sheet, because this part of the insect is composed of several distinct segmentations, as Dennell and Malek (1955) clearly pointed out. The thin intersegmental membrane has an entirely different composition with particular reference to the cement layer and the exocuticle (Dennell and Malek, 1955, Fig. 1, 2. & 4). The abdominal part of the insect cuticle does not give a homogeneous sheet which is required to get satisfactory and reproducible data. This is a very serious matter for the experimental purpose; the data obtained by using sterna or terga can be adopted qualitatively rather than quantitatively. The only data that we can obtain with quantitative reliability is the work of Richards and Fan (1949) and Treherne (1957); in the former case the range of variation per cent((range/mean) X 100) goes up to 30 to 40%, whereas in Treherne's work the per cent standard error ((standard error/mean) X 100) was 20% to 30%. In connection with this experiment, Treherne's work is very interesting: he used the third abdominal tergum of a female grasshopper, Shistocerca gregaria, which resembles somewhat P. americana. In my experiment with the isolated cuticle, the percent standard error was 5% to 10%.

Many reasons for the fluctuation of the data can be proposed: such as contamination by food waste or ecreta in the rearing jar, mechanical damage to the cuticle during the dissecting process, and so on. Nevertheless, at least in the case of the isolated cuticle, the fluctuation is partly caused by water itself, because the permeation rate of water changes with the time of permeation. This idea was confirmed by treating the cuticle with water prior to the penetration test. It has been shown that pre-treatment with water increases permeation in all cases. The difference between the washed and unwashed cuticle decreases with time, and finally reaches a certain constant, which is considered to be due to the washing out effect of water, whereas the suddent increase in the rate of permeation at the early stage is thought to be due to the wetting effect of water.

The theoretical explanation correlates with the change of evaporation of water through the acetone washed cuticle with temperature, but considerable deviation was observed in the experiment with the water washed cuticle and the untreated cuticle. This explains that there are some mechanical disturbances in the natural cuticle which prevent water from penetration by natural diffusion. The barrier can be removed partly by acetone, since the

curve of acetone washed cuticle showed the highest linearity. The barrier can be slightly affected by water washing as well.

The main problems in this field are that the cuticle is so delicate and that the data fluctuates greatly. These difficulties have been some of the most important reasons why quantitative researches, with respect to the cuticular permeation, have not been accomplished. The least fluctuation was observed in the dead, intact roaches, and it is advisable to use the intact roaches for further experiments.

Abdominal terga and sterna are not ideal membranes for permeation studies due to their heterogeneity. In further experiments the permeation test was carried out by using either a very small portion of tergum or sternum, or the cuticle from some other part of the body.

3. PERMEABILITY OF THE INSECT CUTICLE TO MALATHION.

3.1. Introduction.

The mode of penetration of insecticides through the cuticle is of particular interest because of the peculiar facility with which it takes place. For instance, DDT behaves as if the cuticular barrier were not there, since the lethal dosage for contact application scarcely exceeds that established for injection (Brown, 1956). Nevertheless, previous studies in this field have been mostly concerned with the barrier action of the insect cuticle to the entry of insecticides. The problem must be concerned with why insects cannot resist the entry of insecticides. If natural diffusion is taking place in the cuticle, and if the diffusion constant is simply high, the problem is not too difficult. On the other hand, if the penetration deviates significantly from that of natural diffusion, then some factors concerning cuticular composition must be involved. Thus basic understanding of diffusion through the cuticle as a physico-chemical membrane is required.

3.2. Methods and Material.

3.2.1. P-32 labelled malathion synthesis procedure.

As it has been proved that malathion is metabolized in the insect body to a hydrolyzed product which cannot be detected by colorimetric method (Cook et. al., 1958; Cook and Yip. 1958), it was necessary to use another method to measure the exact amount that penetrated through the cuticle. For this purpose a radioisotope was chosen.

Three millicuries of P-32 in terms of phosphoric acid, which was obtained in HCL solution, was used for preparation of radioactive malathion (Casida, 1959; O'Brien, 1958). The product had an activity of 31.8 c/min./gamma of malathion.

3.2.2. Administering and counting procedure.

I have proved that the rate of penetration through the pronotum was constant as was required in these quantitative experiments (Table 9, 10). On the basis of these results, the radioactive malathion in acetone solution was topically applied on the pronotum of P. americana, and to avoid damaging the cuticle, the tip of a needle (Yale BD-25) was cut vertically to the microsyringe, so that 0.02 mm^3 of malathion in acetone could be applied. Intact insects, dead or alive, were used in this experiment. This small

amount of acetone evaporates within a few seconds and the malathion is left on the pronotal plate of the insect. At a given time (e.g. Table 13) the surface was washed out with 10 ml. of acetone, and the amount of malathion left on the pronotum was measured in terms of the radioactivity of the acetone solution. The result was quite satisfactory, since an experiment (Table 14) has shown that the first washing removes more than 97% of malathion left on the surface. The insects were weighed, and the wings were removed before application of malathion. In some experiments the insect body which was washed with acetone was furthermore treated with hot water to extract malathion, which was adsorbed by the cuticle. For this purpose an oven at a temperature of 90°C was used for keeping a constant temperature during extraction. Insects were transfered in a 50 ml. Erlenmeyer flask filled with 10 ml. of hot water, and kept in the oven for given periods. Also in some cases the pronotum was separated from the rest of the body and the amount adsorbed by the cuticle was measured.

For counting the activity, a liquid Geiger-Müller counter with a liquid counting tube (M-6 tube of 20th Centry Electronics Co.), and a scaler (SC-33A of Tracerlab Co.) was used. All material to be counted were dissolved in 10 ml. of

hot nitric acid before counting, The tube has a maximum sensitivity at 1120 volt., and after each counting it was washed carefully twice with acetone and three times with distilled water. Three minutes counts were taken.

Background counting was recorded at the beginning and the end of the experiment and the average was adopted. Since the tube was covered by a lead case, usually the background fell into the range from 30 to 40 counts per 3 min., which was quite satisfactory. In the concentration effect test (Table 19), different concentrations of solutions were used for each administration, so that the volume and hence the area of contact would not be changed.

3.2.3. Radioautography.

In order to study the normal sequence of insecticidal entry insects were allowed to walk in a glass jar which had a malathion residue on the inside walls. Twenty mg. of radioactive malathion in 10 ml. of acetone was poured into a jar of 15 X 15 X 8 cm^3 capacity, and all glass surfaces were wetted by swirling the jar. After the evaporation of acetone malathion was left on the glass surface as a residue. Some amount of grease was put on the top parts of the jar, and the top was covered with a double layer of cheese cloth. A living P. americana was allowed to walk on the surface of the jar. At a given time the insect was taken out and washed by 10 ml. of acetone for 1 min. The body parts were dissected, and all soft and wet prts were removed. A portion of cuticle was put on X-ray film with the endo-cuticle facing the film. The cuticle and the x-ray film were held tightly by two plates of glass being tied with strings, and the whole sample was left in the darkness for two weeks. After that period, the x-ray film was developed by using Kodak microdol developer at 24'C for 10 minutes.

3.2.4. Paper chromatography.

To prove the presence of malathion, a chromatographic technique was adopted according to a method developed by Cook (1954). To a filter paper (Whatman No. 1, 10 x 20 cm), 4 ml. of mineral oil which were dissolved in 100 ml. of ethyl ether were sprayed as an immobile solvent. The sample which was taken from the synthesized malathion in chloroform was applied with a capillary tube to a spot 3 cm. from the bottom of the sheet. Standard malathion was put as well. A mixture of ethanol, acetone and water (1; 1; 2; v/v) was used as a mobile solvent. After developing the chromatogram the spot where malathion was located was detected by means of fluorescein and Nbromosuccinimide.

3.3. Preliminary Studies on Malathion Penetration.

3.3.1. The effect of various treatments of the cuticle on the rate of penetration of malathion.

To know the nature of the penetration of malathion into the cuticle, various initial treatments were applied to the intact cuticle and their effects on penetration by malathion were measured in terms of per cent recovery.

In the case of starved insects the roaches were kept for 2 weeks without food in a clean jar. Water washing was conducted for 2 min., shaking with 10 ml. of distilled water in a 500 ml. beaker. The same procedure was adopted for acetone washed insects, but washing was continued for 5 sec. To another sample of the insects abrasion treatment was conducted with 30 complete back and forth movements by using talc powder and moving the insect on a filter paper. During this treatment the insect was pressed to the filter paper gently. The length of each complete back and forth movement was 10 cm.

Living insects showed more malathion intake than dead insects (Table 6). The difference is regarded as due to the physiological difference between living and dead insects.

The physiological details are not discussed here, but the difference could be established by the physiological effect of the insecticide itself on the physiology of the insect; i.e. the penetration of a similar, but non-toxic compound could be quite different. A very high dose was used for this experiment, because of the low specific activity of the radioactive malathion; and so it was impossible to carry out the experiment without changing the physiological condition of the insects. In the case of starved insects, a relatively high penetration rate was observed.

As mentioned before, the physiological condition of the roaches such as that in starved roaches affects the rate of penetration. However this phenomenon might be due to the mechanical changes in the starved roach, i.e. decreased thickness of the fat body. The effect of the abrasion can be regarded as the effect of the removal of epicuticle, since by such kind of gentle abrasion it has been proven that the main part of epicuticle will be taken away (Wigglesworth, 1945). By water washing, the penetration was also increased over the living control. Penetration following acetone washing may be compared with that of the dead control, since the insect died immediately after acetone washing.

Table 6. The effects of various treatment on the permeability of the cuticle.

Treatment	#of roaches	Total applied	Malath. recovered from body	%Penetration
		c/3min.	c/3min.	%
Living control	5	810	329	40.6
Dead control	5	1293	491	38.0
Living water washed	5	1087	531	48.9
Dead acetone washed	5	1263	497	39.4
Starved living	5	1045	582	55.7
Abraded living	5	937	587	62.6

Temperature 22.0°C
Penetration for 16 hrs.
45 to 48% R.H.
Two females and 3 males.

3.3.2. Further investigations on the effect of water pre-treatment on the permeability to malathion.

In order to investigate the effect of water pre-treatment on the permeability of the cuticle the roaches were washed with distilled water prior to the application of malathion on the cuticle. In this experiment last instar nymphs, which were kept in a clean jar without food until they moulted, were used to avoid any contamination of the cuticle.

It has been shown that water washing increases penetration of malathion in living insects and decreases this penetration in dead insects (Table 7). One might have thought that water affects penetration by removing a water soluble barrier i.e. the washed dead insect should adsorb more than the dead ontrol and the washed living roaches should adsorb more than the living control. The anticipated result was realised only in the living roaches. Clearly then water has not only a quantitative effect, but also a qualitative effect. This phenomenon might be attributed to the water regulatory mechanism of the living insects. In the living insect the cuticle contains a constant amount of water; however, washing the

dead insect with water must increase the water content of the cuticle; and increased water content of the cuticle prevents substances which have a low solubility to water such as malathion from entering.

Thus the increase in penetration of malathion in the living roaches relative to that in the living control may be attributed to the washing out effect, although the increased rate of malathion penetration in dead washed roaches may be largely attributed to the wetting effect of water on the cuticle (see 2.3.3.).

Table 7. The effect of water pre-treatment of the cuticle on its permeability to malathion.

Treat-ments	The rate of malathion penetration (in % of applied malathion)		
	Expt. I 3 males & 2 females 16 hours	Expt. II 4 males 22 hours	Expt. III 2 females 22 hours
Living	40.6%	70.4%	74.8%
Living washed	48.9	76.5	79.1
Dead	----	60.8	46.9
Dead washed	----	54.2	33.5

Only qualitatively reliable. Experimental conditions are not the same in each experiment.

3.3.3. The effect of tanning of the cuticle on its permeability to malathion.

The effect of tanning of the cuticle on the water controlling mechanism of insects has been investigated by many workers; it has been suggested that hard tanning is one of the most im ortant means of water retention in insects. However this generalized idea is not always true; larvae of Tineola are colourless and yet very resistant to desiccation (Mellanby, 1934).

However, the effect of tanning on penetration of insecticides has not been precisely studied. Here an experiment was conducted to study the influence of tanning on the penetration of insecticides through the cuticle.

From a rearing jar, three female roaches were chosen; a freshly moulted and still white roach, a half tanned one, and a completely tanned roach. Two hundred gamma of malathion in 0.02 mm^3 of acetone was topically applied on the 5th abdominal sternum. Twelve hours after this application, the insects were washed with acetone for 1 min., and the rate of penetration was recorded as described before.

The results are shown in Table 8.

The data are to be regarded as qualitative rather than quantitative, since the penetration in the half tanned insect reached 100 per cent. Also the number of insects is not enough to draw a conclusion. Here, however, it can be pointed out that more penetration took place in the incompletely tanned insects than in the completely tanned one.

Table 8. The effect of tanning on the permeability to malathion.

Material (females)	%Penetration	Total counts applied
	%	c/3min.
Completely tanned	82.8	82
Half tanned	100.0	68
Newly moulted (10 min. after molt.)	95.5	89

Penetration for 12 hrs

3.3.4. The application of radioautograph for the study of the cuticle penetration.

The best qualitative method to study penetration is radioautography. A photograph of the extent of insecticidal entry is shown in Fig. 6.

A female of P. americana, was completely submerged into radioactive malathion in acetone solution (approx. 500 gamma per 10 ml. of acetone) for 5 min. After thin treatment the insect was allowed to stand for 5 hours and was dissected. The dorsal part of the cockroach including the pro-, meso-, metanotum, abdominal terga, but excluding the head region and the terminal genitalia, was adjacently attached to x-ray film and allowed to stand for 2 weeks between two glass plates.

It is clear that malathion penetrates different areas of the cuticle, in particular sclerite and membrane, at different rates.

Particular attention must therefore be paid to selecting the part of the cuticle to be studied so that this is homogeneous. The pronotum is suitable. Spiracles seem to have some significance in insecticidal entry; this role of the spiracles was further investigated (Figs. 14 to 17).

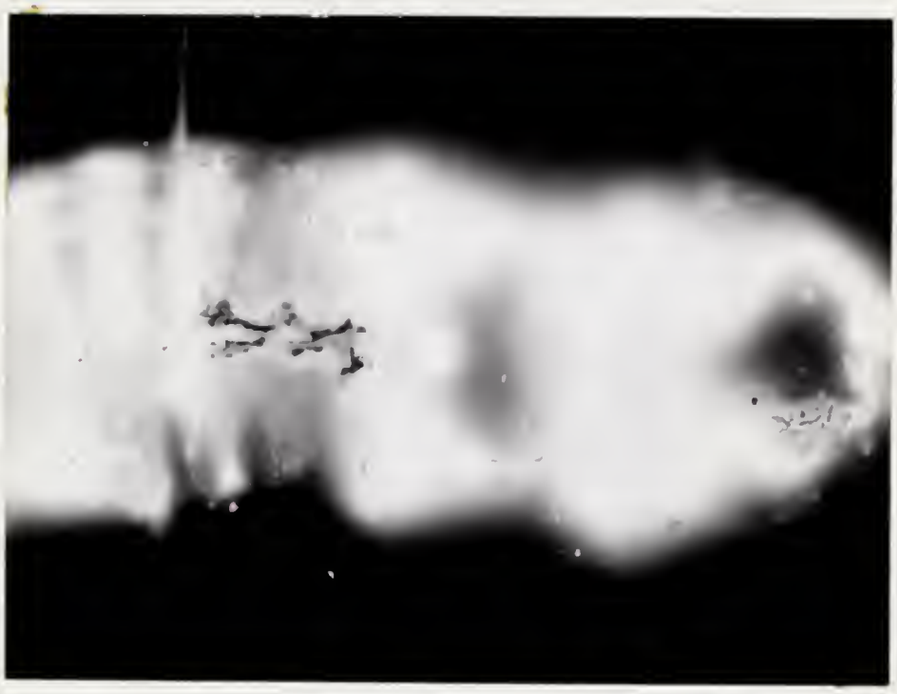


Fig. 6. Penetration of radioactive malathion through the dorsal parts of *P. americana*.

(Note: X 4 in length; pronotum can be seen at the right end of the picture, and the transverse stripes at the center are terga and intersegmental membranes.)

3.4. Mechanical Penetration

3.4.1. Introduction.

The rate of diffusion of material in a homogeneous medium at a certain temperature and pressure is represented by the following equation:

$$\partial c/\partial t = a^2 (\partial^2 c/\partial x^2 + \partial^2 c/\partial y^2 + \partial^2 c/\partial z^2) \qquad (8)$$

where t is time, c is the concentration of the material, a in the diffusivity, and x, y, z, are three dimensional axes, respectively. To make the equation simple, it is usually written:

$$\partial c/\partial t = a^2 \nabla^2 c \qquad (9)$$

Since diffusion through the cuticle can be interpreted into a single dimensional problem,

$$\partial c/\partial t = a^2 \cdot \partial^2 c/\partial x^2 \qquad (10)$$

Here $\partial c/\partial t$ means the diffusion speed in terms of concentration, $\partial^2 c/\partial x^2$ means the changing rate of concent-

ration gradient against the thickness of the membrane x. Now let us assume that the original concentration is very high and constant, and therefore the changing rate of concentration gradient can be decided only by the concentration of the material in the diffusion cell.

Then the equation can be written as follows (Davson & Danniell, 1952):

$$dc/dt - K(Co - Ci) \qquad (11)$$

Where Co is the originally applied concentration, or concentration of the material in the diffusing cell, and Ci is concentration within the diffusion cell(insect body in this experiment). Also K is a constant.

Since the diffusivity constant can be determined as a function of area, size of the diffusion cell, the equation may be written as

$$dc/dt - (PA/V)\ (Co - Ci) \qquad (12)$$

where P is the permeability constant, V is the volume of the diffusion cell and A is the area of contact between the material and membrane.

The equation may be written

$$\int_{0}^{Ci} dC/(Co-C) = \int_{0}^{t} (PA/V)\, dt \qquad (13)$$

Therefore, the equation becomes;

$$\ln \frac{Co}{Co - Ci} = \frac{PAt}{V} \qquad (14)$$

or

$$Co/(Co - Ci) = e^{-PAt/V} \qquad (15)$$

Finally the amount of material penetrated into the diffusion cell(s) is

$$s - Co\ V(1 \times e^{-PAt/V})$$

According to the equation, penetration must be proportional to the concentration of the material administered and exponential to the penetration time; that is, if the penetration follows the natural diffusion rate.

3.4.2. Variations and experimental error.

It is very necessary to obtain precise and accurate experimental values to study the problem quantitatively. In the published data till now, the variation in terms of % standard error is more than 40% (see 1.3.).

In this experiment an effort was made to reduce this variation. A satisfactory result was obtained by using the pronotum instead of adbominal sterna or terga. This choice was made because of the results of the radioautography (Fig. 6): the technique showed that the pronotum was homogeneous in regard to malathion penetration.

Malathion in acetone was topically applied on the pronota of four P. americana according to the method described in 3.2.2., and the penetration rate was recorded in each individual. Variation was studied in terms of % penetration rate. The results are shown in Table 9.

Table 9. The variability of the rate of penetration of malathion through the pronotum.

Wt. of the insects	Malathion remainder on the pronotum	Malathion penetrated	Total malth. applied	%Penetration
1.3477 gm.	1316 c/3min.	671 c/3min.	1987 c/3min.	33.77%
1.1419	1280	755	2035	37.10
1.2837	1124	581	1705	34.07
1.1952	1082	642	1724	37.24

Temperature 23.0°C
Penetration for 1 hr. and 13 min.
45 to 50% R.H.

Average : 35.54

Standard deviation ; 1.88

Standard error : 0.94

%Standard error : 2.64

A similar type of experiment was conducted by using the 5th sternum of a cockroach in order to compare the data with that of Treherne (1957).

The results are shown in Table 10.

About 3 times greater variation in terms of % standard error was recorded in this method compared with the experiment which was conducted by using the pronotum.

In addition, variation can be caused by experimental procedure. As far as the counting procedure is concerned, the probably error expected by the given total number of counts can be dealt with statistically. The calculated % probable error against the total counts is given in Table 11. It is seen that the higher the total counts are, the more reliable they are. Throughout the experiment, the counting material was adjusted to give a range from 200 to 5000 counts at a time. Accordingly the error expected by the counting procedure is to be between 5% to 2% of the value obtained by the experiment. In some cases counts less than 200 were recorded. In such a case its particular % probable error was mentioned in the results.

Table 10. The variability of the experiment of penetration of malathion through the 5th abdominal sternum.

wt. of the insects	Malathion remainder on the sternum	Malathion penetrated	Total malathion applied	%Penetration
gm.	c/3min.	c/3min.	c/3min.	%
1.4350	5,418	4,730	10,148	46.60
1.2146	5,143	5,922	11,065	53.52
1.4263	2,283	2,450	4,733	51.76
1.4396	6,066	3,219	9,285	34.67
1.4213	2,188	2,435	4,623	52.67
1.6144	6,205	4,562	10,767	42.37

Temperature 22.5'C
Penetration for 1 hour.
954 c/3min.; 1 gamma of malath.
45% to 50% R.H.

Average: 46.93

Standard deviation: 7.36

Standard error: 3.01

%Standard error: 6.41

Table 11. The statistical significance of a given total number of counts.

Total counts	%Probable error	Total counts	%Probable error
30	12.27	100	6.74
40	10.65	200	4.77
50	9.54	300	3.89
55	9.07	400	3.46
60	8.70	500	3.01
70	8.06	1000	2.13
80	7.53	5000	0.95
90	7.10	10000	0.67

3.4.3 The effect of time on the rate of penetration.

On the basis of the test of variability, a criterion of the reliability for the quantitative test have been established.

Females wre chosen, and the wings were removed after weighing. The living insects were anaesthesized with CO_2 gas, and held on a dissecting plate with a paper strip by pinning and pressing the both ends of the strip to the paraffin base, so that the insect could not move, or be damaged and malathion was topically applied on the pronotum. The insect was then allowed to stand in the laboratory for a given period. After this period penetration was stopped by washing the surface with 10 ml. of acetone, and the amount recovered in and on the insect was measured in terms of radioactivity. The results are shown in Tables 12 and 13.

It is remarkable that about 10% of the applied malathion penetrates immediately into both living and dead insects. Several reasons could be put forward: (1) it can be suspected that the washing with acetone was not enough, (2) the permeability constant is very high and the short period such as one or two seconds for handling

could affect the penetration rate, and (3) the penetration rate was disturbed by the adsorbing power of the cuticle.

To test the second possibility the permeability constant was calculated in the dead insects. If penetration is taking place according to natural diffusion law, the permeability constant must be independent from time or concentration. In other words, the permeability constant at 10 seconds time of penetration must be the same as the constant at 10 hours later.

The results of these calculations are shown in Table 14.

It has been seen that the permeability constant changes with time. That is, it seems as though the curve shown in Fig. 7 follows the exponential, theoretical diffusion curve. The permeability constant is very high, but not constant with time. This is particularly true near zero; and it tends to a constant value as time proceeds. It is, however, impossible from a consideration of the permeability constant to get 10 % penetration at one or two seconds after administration. I assume that the membrane itself does not change its nature with such a short time hence the high penetration rate in the beginning of the reation must be due to adsorption of malathion or incomplete acetone washing.

Table 12. The effect of time on the rate of penetration in the dead insect.

Wt. of insects	Time	Total applied	Penetrated malath.	Malath. remainder on surface	%Penetration
gm.	sec.	c/3min.	c/3min.	c/3min.	%
0.8282	0	2033	226	1807	11.12
1.0362	30	1671	381	1290	22.80
0.9770	60	2692	777	1915	28.86
0.7300	600	1816	591	1225	32.54
0.9341	3600	1922	610	1312	31.74

Temperature 22.5'C
86.58 c/3min. : 1 gamma of malathion
45-50% R.H.

Table 13. The effect of time on the rate of penetration in the living insect.

Wt. of insects	Time	Total malath. applied	Penetrated malath.	Malath. remainder on surface	%Penetration
gm.	sec	c/3min.	c/3min.	c/3min.	%
0.8083	0	1910	166	1744	9.52
0.7475	30	1805	500	1300	27.98
0.6562	60	1883	712	1171	37.81
0.7449	300	1790	746	1050	41.53
0.7721	600	1835	618	1116	42.33

Temperature 22.3' C
82.5 c/3min.: 1 gamma of malathion
45-50% R.H.

Fig. 7. The effect of time on the rate of penetration of malathion in the dead insect.

Table 14. The permeability constants calculated from the dead intact cuticle (from data in Table 12).

Time for penetration	%Penetration	Permeability constant
sec.	%	cm/sec.
0	11.12	--------
30	22.80	1.28×10^{-1}
60	28.86	7.92×10^{-2}
600	32.54	6.78×10^{-3}
3600	31.74	1.41×10^{-3}

Experimental conditions; see Table 12.

3.4.4. The effectiveness of acetone washing.

The effectiveness of acetone washing of the cuticle must be known in order to measure the amount of penetration of malathion. If the washing does not pick up all of the remainder of malathion on the surface the balance will be recorded as penetrated malathion. Washing with acetone was conducted at zero time with 10 ml. of acetone for 3 min. four times, and the counts were taken.

The results are shown in Table 15.

It can be seen that about 95 to 97% of the remainder of malathion on the surface of the cuticle is taken up by the first washing for 30 sec. The figures have been presented here in terms of % of the remainder on the surface of the cuticle after zero time. Therefore only one to two per cetn of the total amount which is applied on the cuticle cannot be taken off by the first washing by acetone. It seems that, in the cuticle, the wax layer is preventing the adsorption of malathion to some extent, since the extraction of the wax by chloroform increased the rate of adsorption of malathion in the cuticle.

It can be assumed, therefore, that adsorption of malathion by the cuticle could be one of the most important factors which influence the diffusion of malathion into the insect body through the cuticle. It shows that the wax layer is not the main factor of adsorption, and that, if must be beneath the wax layer, since the removal of the wax layer facilitates malathion adsorption.

Table 15. The effectiveness of acetone washing in removing malathion from the surface of the cuticle of P. americana.

Treat-ment	Total applied	% Penetration	1st washing	2nd washing	3rd washing	4th washing
	c/3min.	%	%	%	%	%
Living	1939	11.55	97.02	1.63	1.35	0
Dead	1566	3.93	97.14	1.78	1.05	0
Chloro-form washed	1506	6.71	95.16	3.77	1.07	0

Temperature 22.5' C
74.9 c/3min.: 1 gamma
3 females in each

3.4.5. Adsorption of malathion by the cuticle.

To find the site of adsorption malathion was topically applied on the pronotum and left for 30 seconds in the laboratory condition. The insect was then extracted first with chloroform, then with hot water at 40°C, and finally with 10% of KOH. All extraction procedures were conducted for 30 seconds. The malathion recovery by each extraction is listed in Table 16.

It has been found that the most of the malathion was absorbed in the hot water soluble layer which contains mainly protein as Fraenkel and Rudall (1940) have pointed out. To make sure that the malathion has no impure radioactive component which is soluble in water, the malathion sample was picked up with 20 ml. of chloroform and washed four times with equal volume of distilled water shaking violently, and then the following experiment was carried out.

Four gamma of malathion with acetone was topically applied on the pronota of three females and the insects were allowed to stand for 90 min. The pronota

Table 16. Extraction of malathion from the cuticle.

Material	Total applied	Malathion remainder on cuticle	Chloroform extract.	hot water ext.	KOH ext.	Pene-tration
	c/3min.	c/3min.	%	%	%	%
Living	1567	1339	2.04	8.31	2.56	1.47
Dead	1842	1652	2.33	6.95	0.49	0.59

Temperature 22.5' C
137.1/3min.: 1 gamma
penetration for 1 min.
3 males in each

were washed with 10 ml. of acetone, hot water and chloroform respectively. This time 10 ml. of boiling water were used for extraction putting the container on a hot plate, and the extraction was continued for 2 min. After the water treatment the pronota were extracted with cold chloroform for one minute, and then dissolved in hot nitric acid. the radioactivity of each solution was then measured.

The results are shown in Table.17.

One can calculate that the hot water soluble layer adsorbs 93.14% of the total adsorption of the cuticle and the chloroform soluble layer adsorbs 6.86%. By these extraction methods practically all of the adsorbed malathion can be recovered, and it is clear that the disturbance of penetration of malathion through the cuticle is at least partly due to this adsoprtion phenomenon. There is a proof that adsorption is not due to the impurity of malathion, since malathion was washed with distilled water four times and no activity was transferred into the aqueous phase. It is striking to see that after chloroform extraction, much malathion still remained in the hot water soluble fraction. This suggests that the malathion has a strong affinity to the cuticular protein. The efficiency of hot water extraction was further tested (Table 18).

Table 17. Further investigation of the water soluble layer with regard to malathion absorption.

Total applied (gamma)		Malathion remainder on cuticle	hot water extraction	chloroform extract.	%Penetration	%Recovery in pronotum
c/3min.		%	%	%	%	%
548	(4.0)	6.51	29.74	2.14	29.19	0

Temperature 22.5'C
137.1 c/3min. :gamma
penetration for 90 min.
3 females

Table 18. The efficiency of hot water extraction of the adsorbed malathion in the cuticle.

Total applied	Penetrated	Hot water extraction	Malathion stayed in pronota after extraction	%Efficiency of hot water extraction
gamma	gamma	gamma	gamma	%
56.3	2.96	4.45	0.076	98.21
29.9	1.54	2.76	0.076	96.25
142.9	7.34	8.67	0.400	95.39

Temperature 29.2' C
118.6 c/3min. : 1 gamma
penetration for 210 min.
45% R.H.
Extraction for one hour at 90'C

3.4.6. Further investigation of the effect of time of the rate of penetration.

The effect of time on cuticular penetration was reinvestigated in connection with hot water extraction of the cuticle. The method used in the previous experiment was deopted. The data are shown in Table 19.

The average of the permeability constants from 30 seconds to 240 minutes was 3.376 and its standard error per cent was 6.37. This is a measure of the deviation of penetration from the theoretical diffusion rate.

Here it is seen that malathion enters first into the cuticle and is then adsorbed by the hot water soluble protein of the cuticle. Also it is seen that the penetration rate through the rest of the cuticle closely follows the natural rate of diffusion which can be derived from the theory of diffusion. All malathion entered into the insect body (Fig. 8) shows a similar curve of Fig. 7. Almost all of the adsorbed malathion can be extracted by washing twice with 10 ml. of water: extracted in an oven which was kept at 100'C for 3 min in each extraction.

Table 19. Further data concerning the effect of time on the rate of malathion penetration.

Time	Total malath. applied	Malathion remainder on cuticle	Hot water extract.	%Penetration	%Penetration - hot w. extract	Permeability const. X 10^{-5}
min.	c/3min.	%	%	%	%	cm/sec.
0	1128	94.86	5.14	0.00	5.14	----
30	1211	94.14	5.63	0.23	5.86	2.842
60	1130	92.74	6.29	0.97	7.26	2.975
120	1135	90.84	7.41	1.75	9.16	3.936
180	1168	90.58	7.33	2.09	9.42	3.269
240	1129	90.26	7.35	2.39	9.74	3.862

Temperature 20.5'C
50.8 c/3 min.: 1 gamma
45-50% R.H.

Permeability constant = $(3.376 \pm 0.2151) \times 10^{-5}$

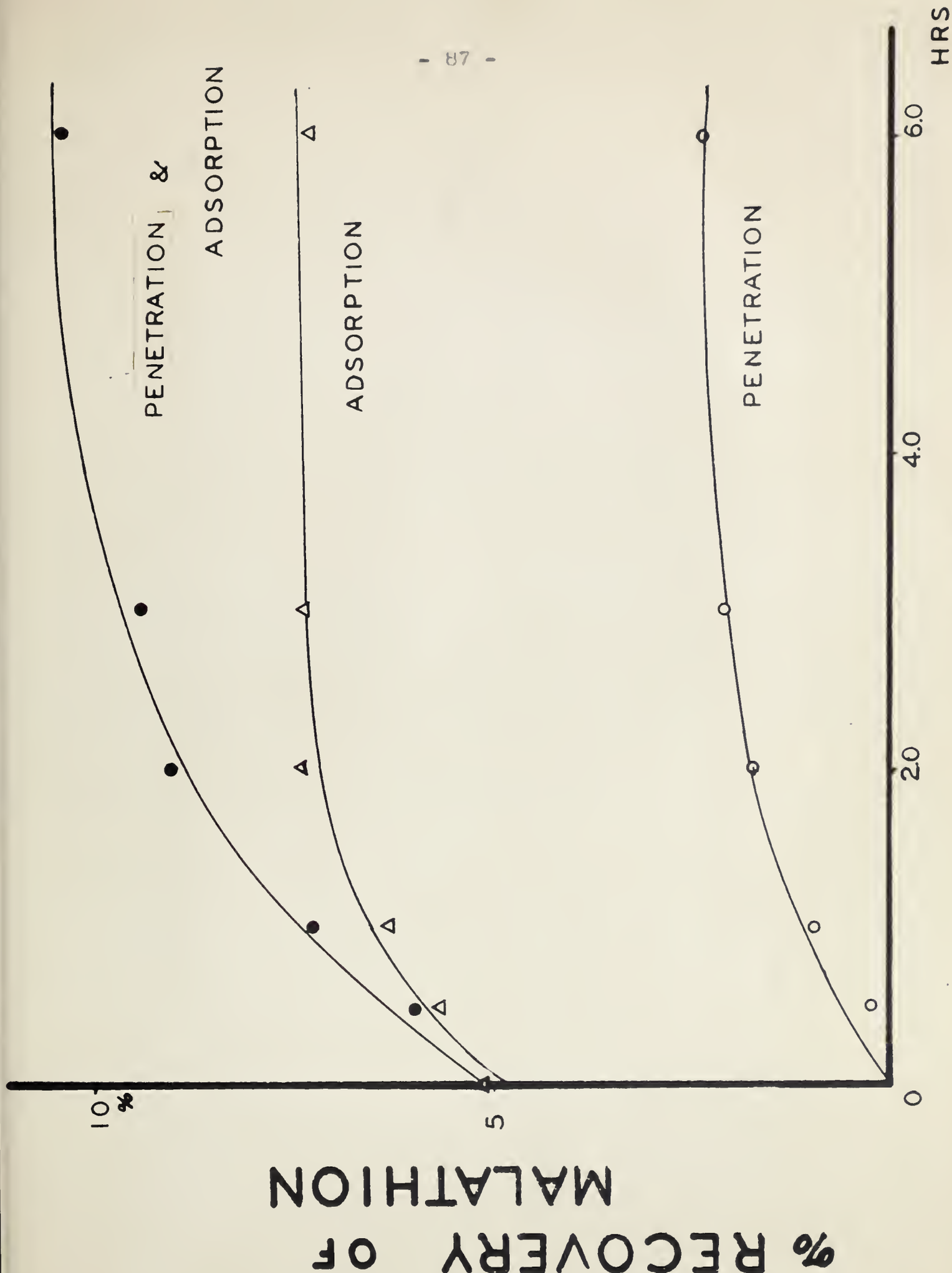


Fig. 8. Adsorption and penetration of malathion into the cuticle against time.

3.4.7. The effect of concentration on the rate of malathion penetration and adsorption by the cuticle.

It can be easily understood from the penetration equation (Eq.16) that penetration must be directly proportional to the concentration of the penetrating material, but there are several types of adsorption. It is true that the penetration rate at higher and lower concentration will not follow the theory, because, to introduce the penetration equation (Eq. 16), it was assumed that the changing rate of concentration gradient across the membrane was proportional to the difference of the concentration between both sides of the membrane. The picture tends to be oversimplified in comparison with the natural phenomenon, since the assumption is true in only a limited range.

Here the experiment was conducted under the assumption that a straight line relationship would be obtained within a limited range, when concentration of applied malathion was varied.

The results are shown in Fig. 9, and Table 20.

Also this assumption might be particularly important with respect to low concentration; with low concentration

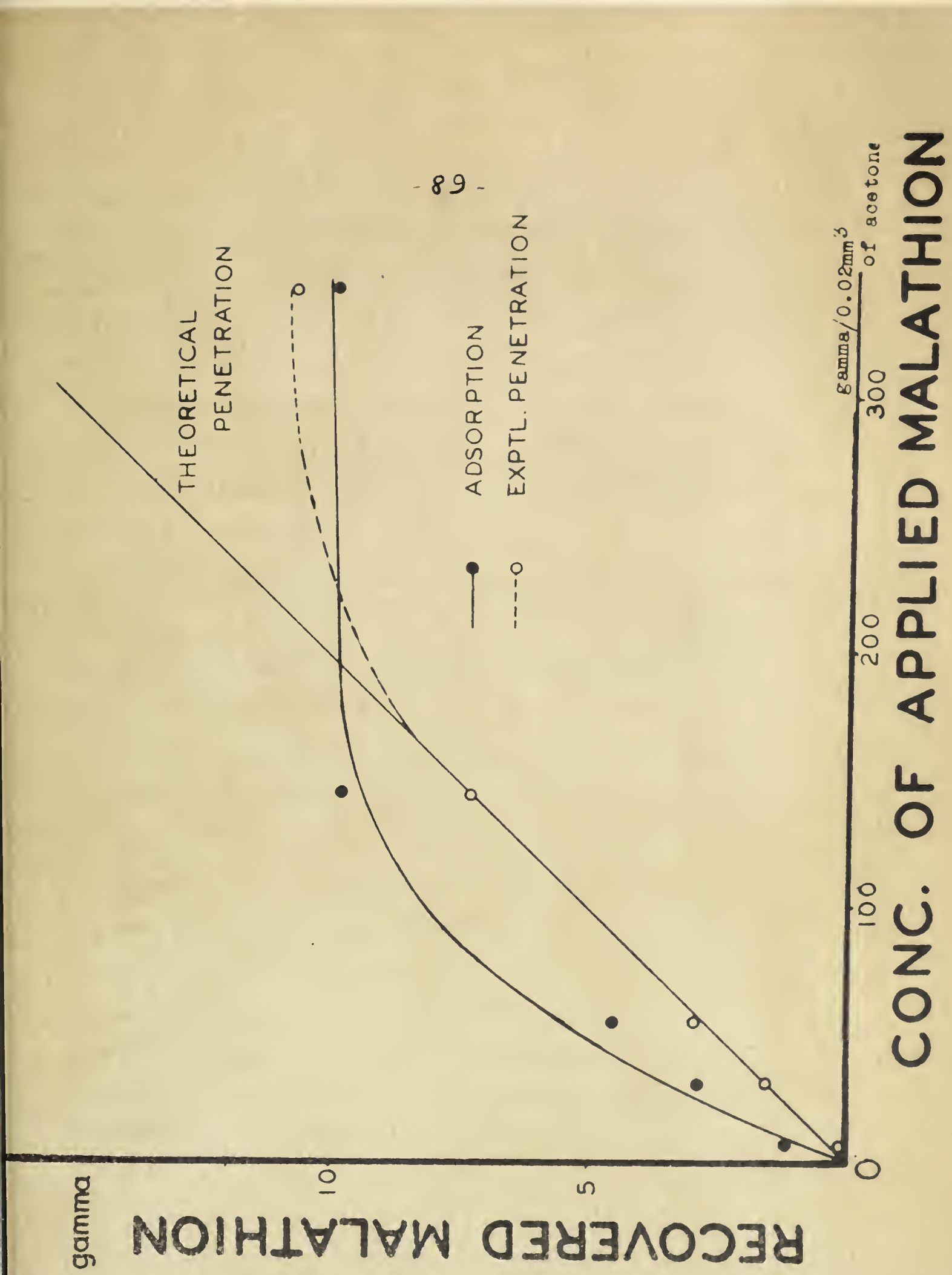


Fig. 9. Penetration and adsorption of malathion against concentration of administered malathion.

the counts obtained are reduced and the probability of introducing errors in terms of counting efficiency of radioactivity is increased. In this experiment, particular attention was paid to keep a constant contacting area between the cuticle and malathion by using solutions of different concentrations each time: otherwise the effect of the applied area might be more than the effect of concentration itself.

Two males were used for each experiment to increase the counts of radioactivity, and therefore to increase the detective amount of malathion. The average of the two is presented in Table 20.

Here it can be clearly seen that the permeability constant slightly deviates in the lowest and the highest concentration test. Since the permeability constant is supposed to be independent of time and concentration, the obtained value can be compared with that of the data from the previous time test. The deviation might be due to the nature of the solution applied on the cuticle; in high concentration the solution tends to show a high viscosity which might prevent the solution from diffusing theoretically. The problem is thus

Table 20. The effect of concentration on the permeability of the cuticle to malathion.

Wt. of insect	Total applied malathion	Penetrated malath.	Adsorbed malathion	Permeability constant X 10^{-5}	%Probable error in counts
0.6962	342.6	10.78	9.18	2.49	2.13
0.6218	142.9	7.34	9.07	3.68	2.55
0.6979	56.3	2.96	4.53	4.23	3.89
0.6668	29.9	1.54	2.84	3.96	4.77
0.6918	5.5	0.10	1.09	3.71	9.54

Temperature 29.2'C
2 males in each
118.6 c/3min. : 1 gamma
210 min. penetration

further complicated by two important factors which cause the rate of penetration of malathion to fall away from linearity. The first factor is the rapid evaporation of the acetone solvent shortly after the application of the drop. This results in a decrease in volume of the initial drop, and hence changes the penetration constant in the penetration equation (16). Now let us assume that the amount of applied malathion (S_o) is

$$c_o v_o$$

where v_o is the original volume of the drop (and is a constant, $0.02 mm^3$ in this experiment), and c_o is the concentration. Since malathion is a liquid at room temperature, the original volume v_o may be expected to be the sum of the volume of malathion v_m and the volume of acetone v_a:

$$v_o = v_m + v_a \qquad (18)$$

When equation (18) is substituted for equation (16), it then becomes:

$$P = \left(\frac{V}{At}\right) \log_n \frac{s_o}{s_o - \frac{(v_m + v_a)S}{v}} \qquad (19)$$

Since v_a decreases to zero as the acetone evaporates, the value of P becomes greater; and so the value of P will be found to become greater at low doses and smaller at high doses of the malathion applied. It was found (see Fig. 9) that the rate of penetration of malathion was higher at low doses but decreased near or at the high levels. This deviation is exaggerated at high dose levels because of the nature of logarithum.

The second factor comes into play when the amount of malathion applied exceeds that sufficient for a monomolecular layer. Since the malathion molecules which are not in actual contact with the cuticle do not have a direct effect on the rate of penetration, an increase of malathion beyond the saturation point (approximately 120 gamma in these experiments) does not increase the rate of penetration. Thus the rate of penetration is expected to fall away distinctly from linearity at doses beyond the monomolecular threshold.

On the other hand, the adsorption of malathion by the cuticle shows very interesting characteristics as shown in Fig. 9. It can be seen that the amount of material adsorbed per given quantity of adsorbent increases relatively rapidly with pressure, or concentration of the material, and then much more slowly as the surface becomes covered with the material molecules. To present the variation of the amount of adsorption per unit area, Freudlich (1916) proposed the following equation on purely empirical grounds:

$$y = K \cdot P^{1/n} \tag{20}$$

Here y is the weight or amount of the material adsorbed per unit area or unit mass of adsorbents, P is the equilibrium concentration, and K and n are empirical constants dependent on nature of the solid and the material at certain temperature. The equation may be tested as follows. When expressed logarithmically, the equation becomes:

$$\log_{10} y = \log_{10} K + (1/n)\log_{10}P \qquad (21)$$

Now if $\log_{10}y$ is plotted against $\log_{10}P$, a straight line should results with the slope equal to 1/n and ordinate intercept equal to $\log_{10}K$. Figure 9 shows such a plot as construction from the given in Table 20. Although the requirements of the equasion are met satisfactorally at the lower concentration, the experimental points curve away from the straight line at higher concentrations, indicating that this equation does not apply when reproducing the adsorption of malathion by the cuticle.

A much better equation for this type of adsorption has been presented by Langmuir (1916). Langmuir first postulated that the material in being adsorbed by a solid surface cannot form more than a monomolecular layer. Further he visualized the adsorption process as consisting of two opposing actions; a condensation of molecules from the material phase onto the surface and an evaporation or backward diffusion of molecules from the surface of the solid.

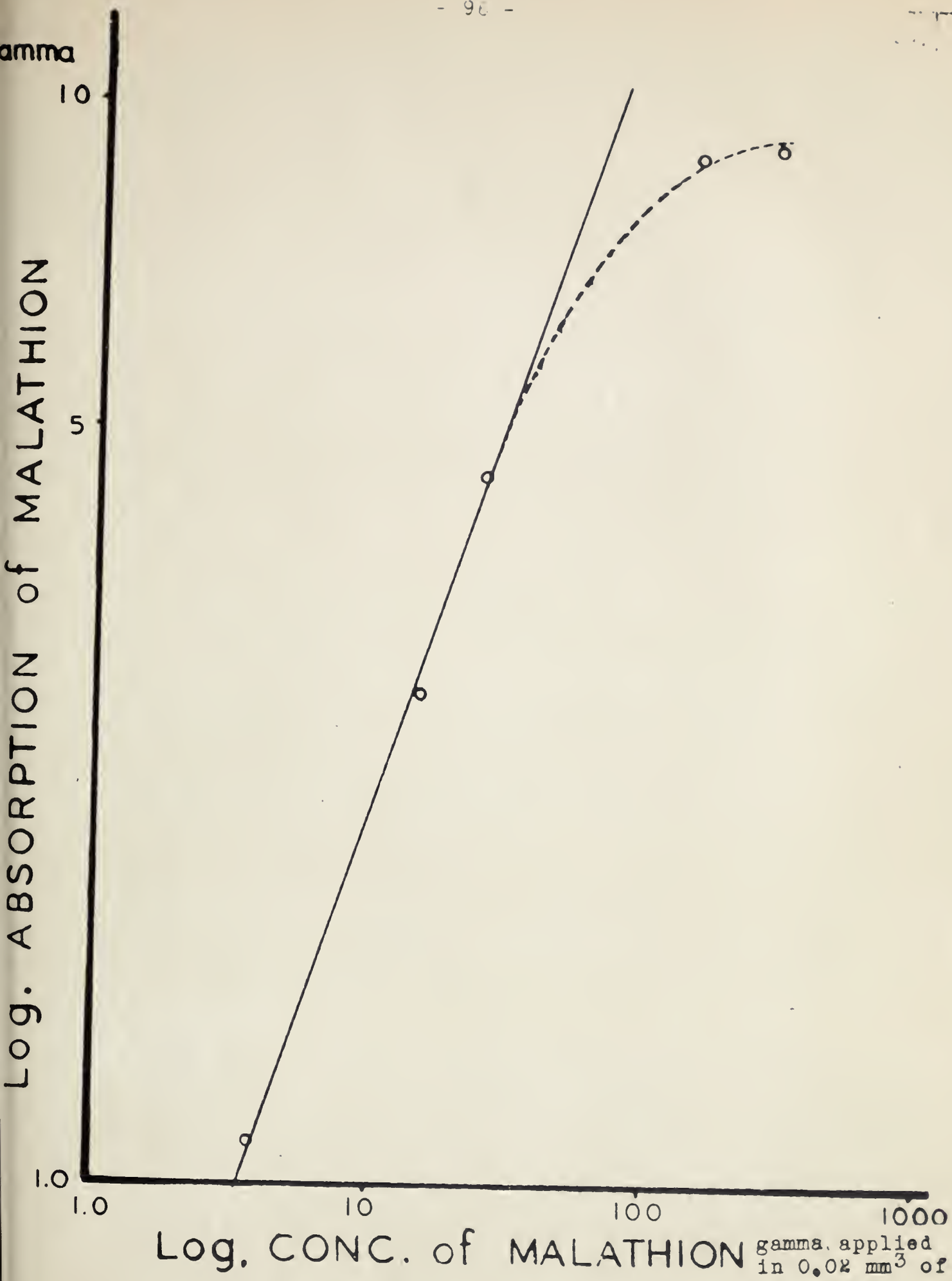


Fig. 13. Adsorption of malathion by the cuticle(The data were plotted according to Freudlich's adsorption equation; see also pp 94& 95 of the text.).

When adsorption first starts, every molecule from the surface may condense on it. However, as adsorption proceeds, only those molecules which are contacting with the area of surface available for adsorption can be adsorbed.

Langmuir's adsorption equation can be written as follows:

$$y = aP/(1 + P \cdot b) \qquad (22)$$

Here a is an empericial constant, b is K_1/K_2, the rate of adsorbing and backward diffusion, y is adsorption, and P is the original concentration of the material. Now the equation can be varified into

$$P/y = (1/a) + (b/a)P \qquad (23)$$

Since a and b are constant, a plot of P/y vs. P in above equation should yield a straight line with a slope equal to b/a and an ordinate intercept equal to 1/a. In Fig.11 such a plot is given from the same data as used in Fig.10.

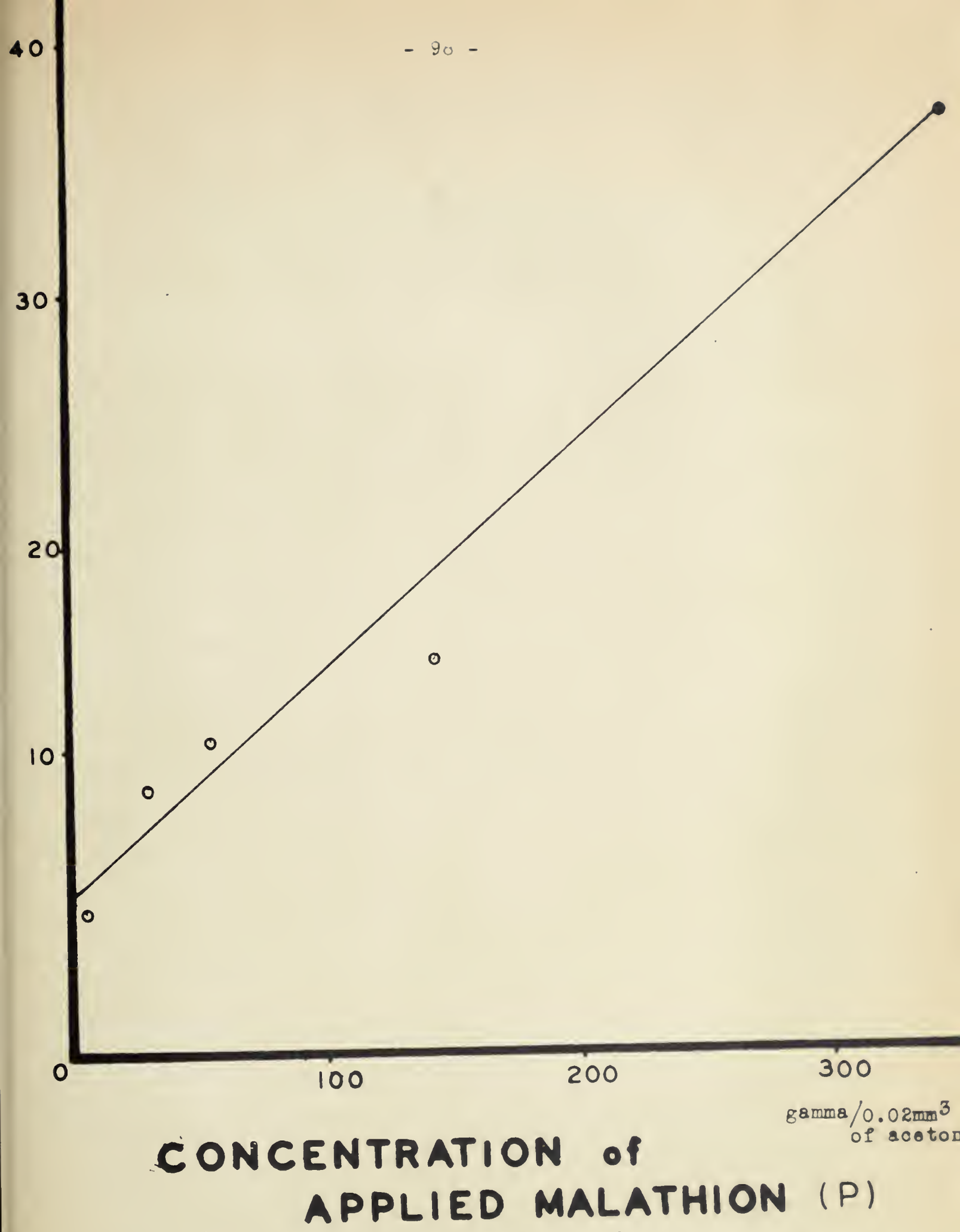


Fig. 11 Adsorption of malathion by the cuticle (The data were plotted according to Langmuir's adsorption equation; see pp 97).

3.4.8. The effect of temperature on the rate of penetration of malathion.

It is very important to know the effect of temperature on penetration, since the effect of concentration or time does not change the permeability constant but temperature affects the permeability itself, and penetration and adsorption show different rates of response to varying temperatures. As described before, if penetration can be independently measured, the logarithm of the amount of penetration must be proportional to 1/T.

The results are shown in Table 21.

The recovery of radioactive malathion from the insect body after acetone washing and hot water extraction is proved to show a theoretical diffusion as shown in Fig. 12. (see Eq. 6).

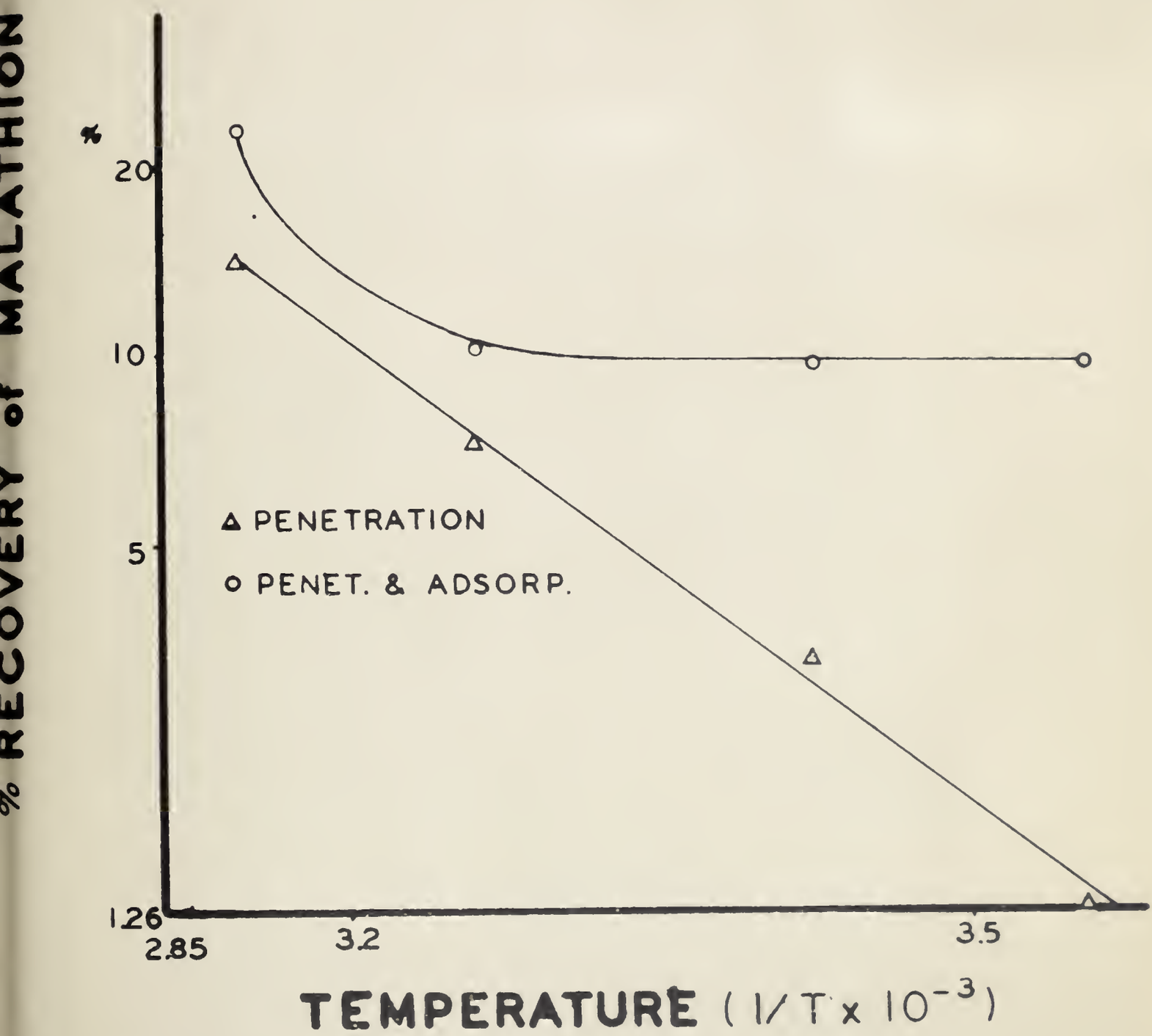


Fig. 12. The effect of temperature on the permeability of malathion through the cuticle.

Table 21. The effect of temperature on the rate of malathion penetration.

Temperature	Total applied	Malathion remainder on cuticle	Adsorption	% Penetration	Adsorption & Penetration
5.5	1010	90.20	8.51	1.29	9.80
23.7	779	90.24	6.55	3.21	9.76
50.0	913	89.28	3.51	7.23	10.74
71.0	999	79.78	8.71	14.11	22.82

Penetration for 10 min.
1 male each
58.75 c/3min. : 1 gamma
60% R.H.

3.4.9. The meaning of tanning and penetration.

Previous studies have shown that penetration in the completely tanned cuticle is lower than the penetration in the newly moulted cuticle, but the phenomenon must be reinvestigated, since by all usual methods the malathion remaining in, as well as that penetrating the cuticle is detected. Therefore, the real nature of penetration must be studied by removing the hot water soluble layer as an absorbent. The results are shown in the following page (Table 22).

To test the adsorption a very short penetration time was used, namely 30 seconds. It was clearly shown that in spite of the high total insecticidal entry into the cuticle, the newly moulted insect did not show higher penetration as compared with that of the darkly tanned insect. In this experiment, a lower penetration was shown in the newly moulted insect but the value itself has relatively low reliability because of its low counting.

The data may be compared with that of Fraenkel and Rudall (1947). According to them the water soluble, particularly cold water soluble layer, which is largely

Table 22. The effect of tanning on the rate of penetration and adsorption of malathion.

Insect	#	Total applied	Cold water extract	Hot water extract	%Penet-ration	Total entry
		c/3min.	%	%	%	%
Newly moulted	3	1913	5.65	3.92	0.16	9.73
Darkly tanned	3	2069	1.59	1.01	0.39	2.99

Temperature 21.1'C
48.4 c/3min. : 1 gamma
Penetration for 30 sec.
females

composed of protein, decreases as the tanning proceeds, and the rate of change at this stage is 33% in the newly moulted cuticle and 8% in the darkly tanned cuticle. Experimentally (Table 22), it has been shown that the adsorbed malathion recovery by means of hot and cold water extractions is 9.57% in newly moulted P.americana and 2.60% in darkly tanned ones. Therefore, the cuticle of a newly moulted roach adsorbs four times as much as that of a darkly tanned one. Now Fraenkel and Rudall claim that the newly moulted roaches have four times as much water soluble cuticular protein as have darkly tanned roaches. Essentially these author's data agree with those of the present experiment with respect to the amount of malathion adsorbed by the cuticle. Therefore it can be reasonably assumed that adsorption is taking place at the water, or not water soluble layer in the cuticle, and that this layer must be the protein containing layer in the endocuticle as Fraenkel and Rudall have reported.

3.5. Physiological Penetration.

3.5.1. Introduction.

In previous chapters the cuticle permeability was studied with respect to the physical and chemical interaction between the substance and the insect cuticle. The biological factors must be considered as well. It is very important to know the site of insecticidal entry under natural conditions. For example, Roy (1944) has noted with regard to pyrethrum that most of this insecticide enters by way of the spiracles instead of passing through the cuticle. Therefore, a precise study of how much malathion enters by way of the spiracles and the rate of spiracular penetration as compared with cuticular penetration is necessary.

3.5.2. Penetration in living insects.

It has been found in this series of experiments that the penetration rate in living insect is always higher than that in dead insects (Tables 12 & 13). However, the reason is not clear. It could be a mechanical difference or a physiological difference such as the effect of the blood stream or the activity of the enzyme system. There is a very important factor to be considered in penetration studies in living animals. If the penetrating substance is non-toxic to the insect system, then natural penetration occurs, but as insecticides are toxic, it is theoretically impossible to get similar results as those obtained with the use of non-toxic matieral. In order to prevent mortality, the amount of insecticide applied must be as small as possible, or the radio-activity counts of the insecticide must be as high as possible.

Table 23. The comparison of the insecticidal penetration of living and dead insects.

Time of penetration	%Penetration in dead 22.5'C	%Penetration in living 22.3'C
sec.	%	%
0	9.52	11.12
30	22.80	27.98
60	28.86	37.81
300	----	41.53
600	32.54	42.33
3600	31.74	-----

86.58 c/3min. : 1 gamma in dead
82.5 c/3min. : 1 gamma in living females.

The rate of penetration in dead and living insects can be compared in terms of time effect (Table 23). It is easily seen that the difference in the amount penetrated does not change after a certain period of time; for instance, the difference at 60 sec. is 8.96% and that at 600 sec. is 9.75%. The relation can be clearly seen in Fig. 13. The curve represents two closely parallel lines. This suggests that the difference is not due largely to the mechanical composition of the insect cuticle, but is possibly due to the physiological difference giving an absolute difference value between dead and living insects.

To obtain more data, the hot water extraction procedure was carried out (Table 24). It can be pointed out that malathion adsorption by the cuticle does not vary greatly in the two cases; but the amount penetrated into the body is much higher in the living insect than in the dead.

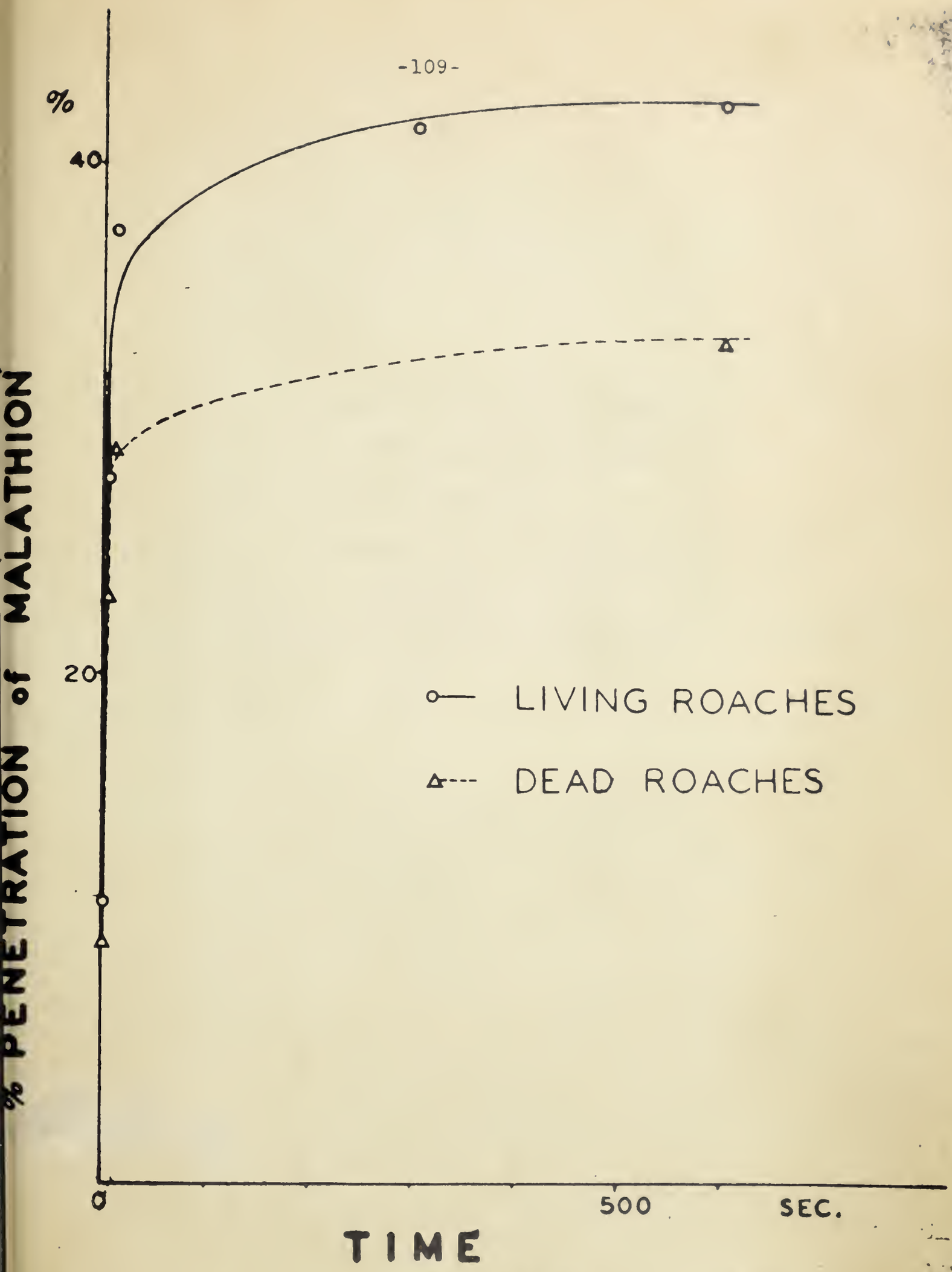


Fig. 13. The effect of time on the rate of penetration

Table 24. The comparison of the insecticidal penetration in living and dead insects with hot water extraction

Insect	#	Total applied	Hot water extract	%Penetration
		c/3min.	%	%
Living	2	520	3.27	8.65
Dead	3	483	4.08	5.86

Penetration for one hour
39.9 c/3min. : 1 gamma
22.5'C
Extraction for 2 min. at 80'C
females

3.5.3. The site of the insecticidal entry. - The role of spiracles.

The role of the spiracles in reference to insecticidal entry seems to have been overemphasized. Nevertheless, this phenomenon has to be reinvestigated in the case of the newly developed contact insecticides such as organic phosphorus compounds.

A female cockroach was allowed to walk for three hours on the surface of a glass jar on which 40 mg. of malathion, the specific activity of which is 41.8 c/3min./gamma at a temperature of 22.5C., were placed. After the given time, the insect was transfered into a beaker and was washed thoroughly with 10 ml. of acetone for a period of 3 minutes. Since the amount which affects the insect is the absolute amount and not the amount per unit weight of the insect tissue, the parts of the insect were weighed but the amount per unit weight was not presented (Table 25). After 3 hours of exposure the insect was "knocked down" and showed convulsion symptoms. The insect was carefully

Table 25. Penetration and distribution of malathion in a living insect.

Organs	Malathion penetrated & recovered	Weight of organs
	c/3min.	mg.
Tracheal system	272	3.8
Cuticle	3938	284.8
Head and antennae	947	53.2
Legs	11700	283.1
Intestins & fat tissue	1173	255.2

Temperature 22.5'C
Penetration for 3 hours.

dissected under a microscope and counts taken. The results are shown in Table 25. The amount of malathion detected was particularly high in the legs and in the cuticle but a minimum amount of activity was detected in the tracheal system. Therefore, it can be inferred that the tracheal system is not the main point of entry, and is not the principle site of action of malathion. However, the assumption cannot be established unless the time factor is considered. That is, since the specific activity of malathion in the tracheal system is much higher than the activity in the digestive system and fat body, malathion might be entering through the trachea continuously. To study the actual sequence of insecticidal entry, roaches with closed spiracles were compared with those in whcih the spiracles were open. The spiracles were closed by means of rubber cement. Also, to maintin the spiracles of the control open, the roaches were treated with carbon dioxide before they were placed into a jar which had previously been sprayed with malathion. The results are shown in Table 26.

First it can be seen that there is no significant difference between insects with closed spiracles and the

Table 26. The role of spiracles in insecticidal entry.

Treatment	Time exposed	Remainder on the cuticle	Adsorbed by the cuticle	Penetration	% Penetration through spiracles
	min.	c/3min.	c/3min.	c/3min.(gamma)	%
Spiracles closed	30	1148	731	133(1.43)	------
Spiracles open	30	1739	769	156(1.68)	14.74
Spiracles closed	120	1699	1637	421(4.53)	------
Spiracles open	120	1716	1589	471(5.07)	10.62

Hot water extraction for 90 min. at 90'C

Males
Temperature 24.0'C
92.95 c/3min.: 1 gamma

control insects. In the first 30 minutes about 15% of the malathion apparently entered through the spiracles. In addition, this value is supposed to decrease in insects with closed spiracles: the activity in insects with closed spiracles is much less than that in the normal ones. It is very interesting that the penetration through these organs is more important in the first step of penetration than in the succeeding steps. However, if the absolute value of malathion is taken, only 0.25 gamma out of 1.68 gamma of penetrated malathion entered through the spiracles in the first 30 minute period. This amount is probably not fatal to a cockroach. In this experiment the amount of malathion extracted from the cuticle is extremely high as compared with the data obtained previously. This might be due to the fact that the whole body, including the legs which have been found to have a very high adsorbing capacity, is responsible for adsorbing the

insecticide, whereas in the previous experiment only a portion of the prothorax was used. This fact is particularly important in nature, since the period during which the insect is exposed could be very short but the amount of insecticide taken up by the insect must reach the lethal dose. It can be pointed out that the amount recovered from the cuticle as adsorbed malathion might be fatal to an insect even after it has escaped a treated area. As a result of this experiment, it can be concluded that a large amount of malathion enters the insect body through the cuticle, and the adsoprtion of malathion by the cuticle increases the rate of entry. On the other hand, 10 to 15% of the insecticide enters through the spiracles.

3.5.4. The radioautographic method for studying the site of insecticidal entry.

The previous study concerning the site of entry of malathion does not describe precisely where the insecticide enters and how much enters at a certain part of the cuticle, In the following experiment the insecticidal

entry was photographed using the radioautographic technique. The same method of preparation as in the previous chapter (3.3.4.) was used. After being exposed, the insects were dissected without using water. The malathion residue on the surface of the cuticle was removed by washing with 10 ml. of acetone for 1 min. Then the insect was dissected and the soft and wet parts of the body were removed completely. In this way only the adsrobed malathion was left within the cuticle. After careful removal of the wet tissue, the cuticle was allowed to dry for about an hour. Then the material was put between two glass slides with an X-ray film sheet. The results are shown in the following figures, (photographic positives). The white part of the film shows the presence of P-32 malathion in the cuticle.

After being exposed for 3 minutes, the insect seemed to be affected only at the tips of the antennae, as shown in Fig. 14. The tip of the antennae were found to be exposed and very sensitive to malathion.

The cuticle of the legs was also particularly sensitive to the insecticide, but photographs are not presented since they were spoiled in the developing process.

At the 5 minute stage the insects began running around very violently on the walls of the glass jar.

At the 8 minute stage three roaches began a cleaning action with their antennae and one also showed locomotive instability and tremor of the legs. The cleaning of the antennae was continued for about 20 to 30 minutes and at the 12 minute stage the insects stopped running.

At the one hour stage all insects were knocked down but they made attempts to get up. Two of them succeeded until such time that they were knocked down again. In the photograph of the insect exposed for one hour, it can be seen that the tips of the antennae and the tibia and tersi have absorbed a large amount of malathion (Fig. 15).

Fig. 14. Radioautography of malathion penetration for 5 minutes.

(Note: X 2.5 in length; the tips of the antennae can be seen at the right end of the picture.)

The mouth parts were also dissected and radioautographed. As can be seen in Fig. 15 malathion had been adsorbed in the tips of the maxillary palpi. This was probably caused by the antennal cleaning action. However, the effects of malathion with respect to these structures was not found to be as great as supposed before. There are certain pitfalls evident with the decision that the cleaning action causes oral intoxication and that the oral intoxication is the main way in which the insect is killed. In this experiment it has been shown that large amounts of malathion were not adsorbed by the mouth parts.

In the case of the dorsal part of the insect, the photograph is not clear. The area which shows a clear boundary is the part spoiled by the soft tissue attaching to the X-ray film and causing decomposition of the film. It can be seen that the edges of the abdominal sclerites are more sensitive than the other parts of the abdomen. It seems, however,

Antennae

(Note: X 2.5 in length; the tips can be seen at the left of the picture.)

Legs

(Note: X 2.5 in length; the fore leg(left) and the middle leg(right).)

Fig. 15. Radioautograph of malathion penetration for one hour.

Mouth parts: Labium and maxilla

(Note: X 3 in length; the tip of labium (upper) can be clearly seen as a white spot.)

15 a., Penetration for one hour(Continued).

Dorsal parts (Note: X 2.5 in length)

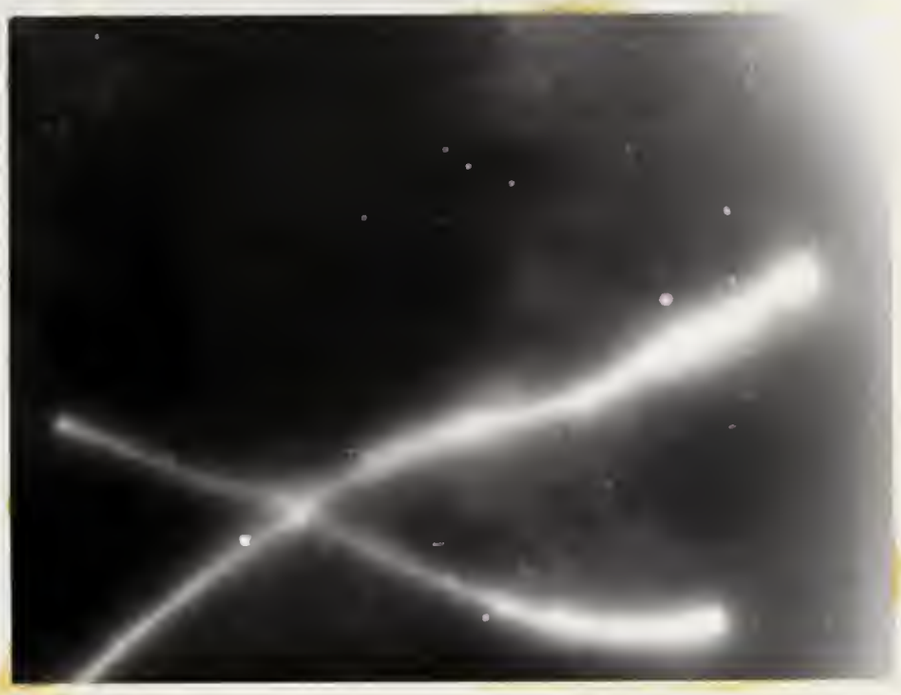
Abdominal sterna

(Note: X 2.5 in length; the clearly exposed part at the right corner corresponds to the 1st sternum.)

g. 15 b., Penetration for one hour (continued).

that the abdomen and the thoracic parts of the body are not too important in connection with the insecticidal entry.

At the three hours stage, all the insects were knocked down completely and showed violent convulsions. The joints of the legs and the mouth parts were found to be affected (Fig. 16). The results are striking, when compared with the deposition of the insecticide at the same part of the insect at the one hour stage. A sudden increase in the amount of deposition at the three hour stage indicates that oral intoxication by means of the cleaning action of the insect is also important at the later stages of intoxication. In this stage it can be also observed that the deposition of the insecticide on the dorsal parts and the sterna has increased. The central parts of the thoracic and abdominal tergites were not affected because they are shielded by the wings. The sterna did not show much deposition, but the edges again were found to be more sensitive to the entry of malathion.



Antennae

(Note: X 2.5 in length; the tips of the antennae can be seen at the right end of the picture.)

Fig. 16. Radioautograph of malathion penetration for three hours

Legs

(Note: X 2.5 in length; the hind leg (upper) and the fore leg (lower and right hand) can be seen. At the left of the picture the hind femur was shown.)

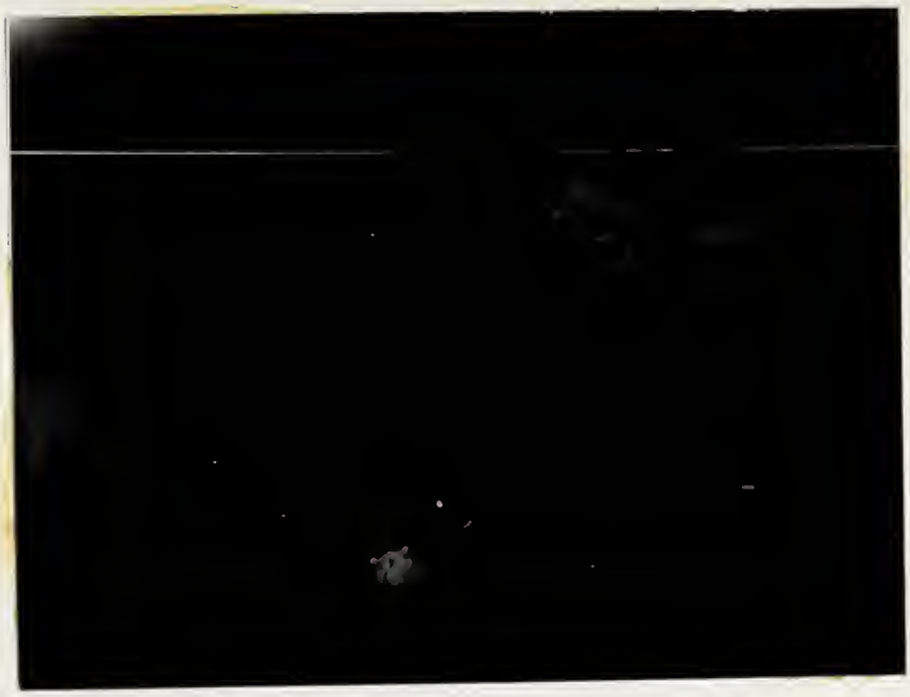
g. 16 a., Penetration for three hours (Continued).

Maxillary palpi

(Note: X 5 in length; the right maxilla)

16.b., Penetration for three hours (Continued).

(Note: X 2.5 in length; at the right hand the pronotum can be seen as a round plate.)
Dorsal part



Abdominal sterna

(Note: X 2.5 in length)

16 c. Penetration for three hours (Continued).

At the ten hour stage, the insects did not show violent convulsions, but were twitching every five to ten minutes. Excluding the twitching, the insect did not show any other movement. The antennae and the legs showed a tremendous amount of deposition as shown in Fig. 17. The prothorax also showed a large amount of contamination which decreased toward the center of the prothoracic plate. The central parts, therefore, proved to be the most resistant to adsorption.

Antennae

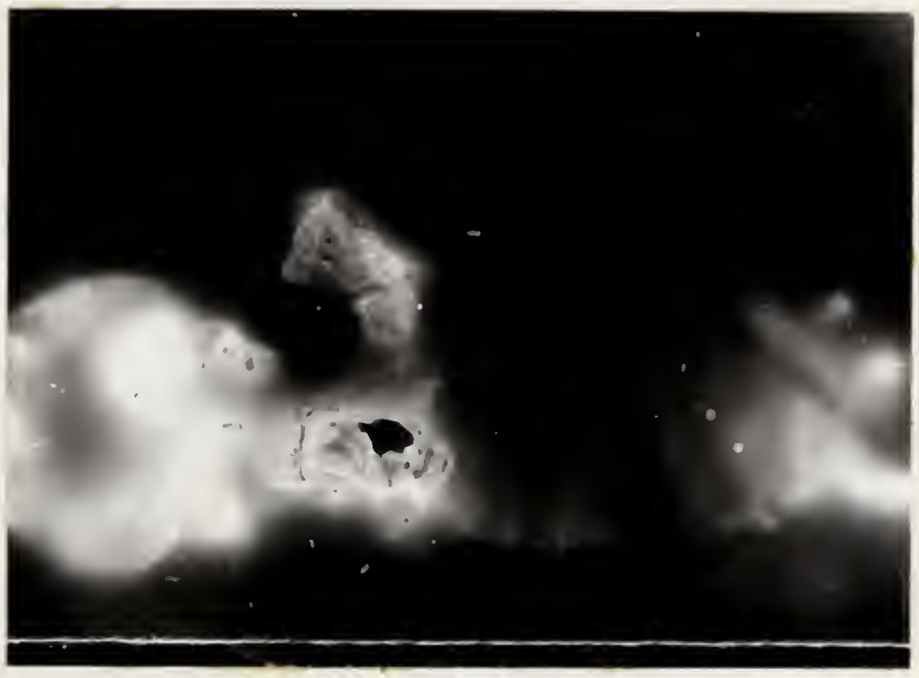
(Note: X 2.5 in length; the tip can be seen at the right end of the picture.)

Fig. 17. Radioautograph of malathion penetration for 10 hours.

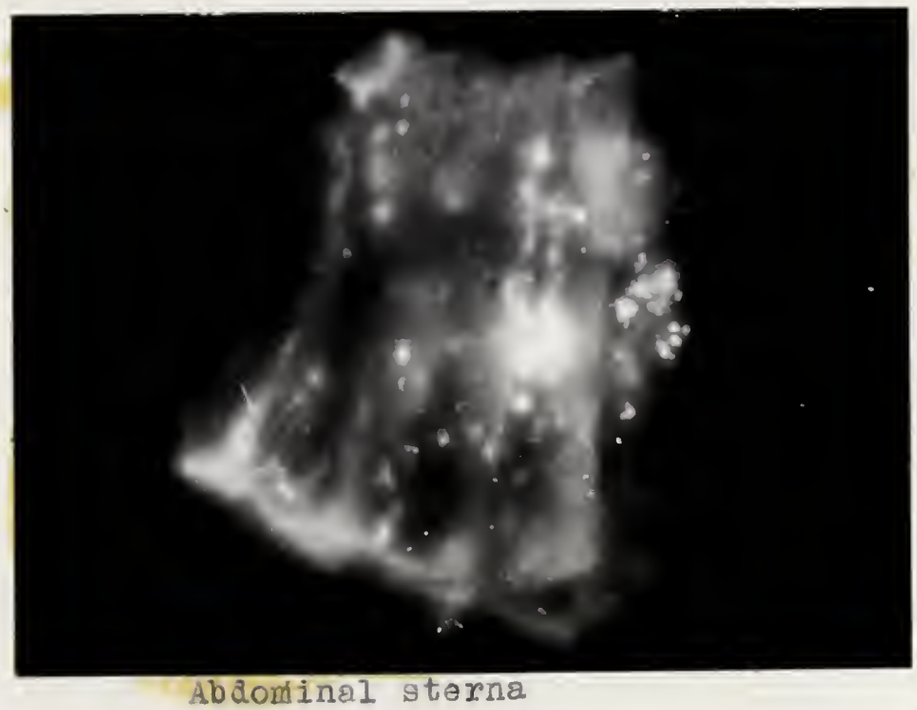
Legs

(Note: X 2.5 in length; the fore leg (left) and the hind leg (right) are shown.)

Fig. 17 a., Penetration for 10 hours (Continued).



Dorsal part



Abdominal sterna

(Note: X 2.5 in both cases; the round plate at the left hand of the upper picture is the pronotum. It can be seen that the central part of abdomen was not exposed, due to the presence of wings(upper).)

Penetration for 10 hours (Continued).

4. DISCUSSION

It has been shown that the penetration of malathion through the insect cuticle does not follow a purely theoretical equation, but the amount which actually enters the insect body is much more than that assumed from the theoretical point of view. The deviation is particuarly high at the beginning of penetration. This fact led me to assume that malathion enters the cuticle not only by penetration but also by adsorption. Also it has been found that adsorption occurs at the hot water soluble layer of the cuticle. If the adsorbed malathion is removed, the rest of the malathion which entered the insect body follows a simple diffusion equation. However, the permeability constant tends to decrease at the first step of penetration: from 0 to 60 min., as can be seen in Table 19. This phenomenon can be explained in connection with adsorption; before the layer gets saturated, the rate of penetration is limited by the fact that some of the molecules are used to saturate the layer.

Adsorption has been shown to be complete after 60 min. of penetration (Fig. 8). The penetration constant which was obtained in this experiment is relatively high when compared with the data of Treherne (1957); in his experiment the permeability constant is in the order of 10^{-6} to 10^{-7}, whereas in this experiment the value is in the order of 10^{-5}. The difference is possibly due to the design of the experiment. Treherne (1957) used isolated cuticle and a diffusion apparatus.

The intact insect body is not an ideal diffusion cell; it is not homogeneous, and the malathion which diffuses into the body is taken up only by particular tissues.

Now the penetration equation to obtain the permeability constant is

$$P = (V/At)\log_n(Co/(Co-Ci)) \qquad (24)$$

The details of the equation have been explained in a previous chapter (3.4.1.). Here V is the volume of the diffusion cell through which the material will penetrate. In this experiment, the volume of the insect body was adopted as V in order to obtain the permeability constant. However, the real size of the diffusion cell into which the material diffuses is much smaller than the whole body. For instance, the tissue which is directly concerned with the permeability itself might be the layer adjacent to the cuticle or it might be also the blood of the insect. It would not be too surprising, if the value is found to be one tenth of the body volume. The actual volume of the diffusion cell can be obtained by means of a comparison of the data of the permeability constant in the intact insect and in the isolated cuticle. The difference obtained will provide the difference in volume of the diffusion cell (insect body) and the <u>diffusible intact volume</u>, which is the actual volume being utilized for diffusion.

It is very important to mention that the main object in this experiment is not to obtain the permeability constant. The purpose in calculating the permeability constant is to find how much the rate of penetration deviates from that in theory. The permeability constant must be constant despite differences in time and external concentration of the penetrating material. The deviation from theoretical diffusion explains the absorption phenomenon therefore,

With respect to the variation in the data, the experimental results were acceptable; in four experimental trials using four cockroaches in each trial the standard error was proved to be less than 3%, whereas that of Treherne was about 5 to 20%, and that of Richards and Fan (1949) was more than 2 to 18 times of the lowest value of the experiment. The low rate of variation obtained in my studies might be partly due to the mode of life of the insect and also partly due to the portion of the insect body that was used.

By using the fifth sterna instead of the pronotum of P. americana, a higher per cent standard error, 6.41%, was obtained. This seems to be due to the heterogeneous nature of the abdonimal cuticle, which is evident in Fig. 16. Richards & Fan (1949) obtained a high variation and yet the variation does not seem to be because of experimental error, but because of the nature of the cuticle itself. The cuticle is not homogeneous to penetration of various substances. They used an 8x10 mm sheet of a larval cuticle, but it has been shown by radioautographic methods that to obtain a homogeneous sheet of this size is almost impossible , as far as penetration of malathion is concerned.

In this experiment, it has been shown that water affects the nature of the cuticle. The cuticle was usually washed and dissected in water in previous workers' experiments, and the soft tissues were removed with a cotton swab. This type of treatment is harmful to the endocuticle where the water soluble layer is located. Treherne's explanation stating that the variation is due to the contamination of the cuticle by food waste

and excreta in the rearing jar is one of doubt. This statement is based largely on the fact that the contact angle of a water droplet changes when the cuticle is previously immersed in water (Holdgate, 1955). Nevertheless, Holdgate did not differentiate between the wetting effect and the washing out effect. The change in the contact angle after water immersion is very high in P. americana (Holdgate, 1955. Fig. 3), and it is assumed that this same degree of effect could be introduced by exposing the cuticle to high humidity conditions. As far as these experiments were concerned, no practical differences regarding insecticidal penetration was obtained from the insects which were reared in a clean jar and those normally reared.

Before comparing the permeability constant with that of other workers, it is necessary to mention the difference of the method of insecticide application and its effect on the permeability constant. As mentioned before, in the topical application the definition of concentration is not clear. That is, the solvent applied with the material evaporates very rapidly, and after a time only the given material will be left on the cuticle. The concentration change is higher in the dilute solution

and smaller in the highly concentrated solution. Concentration does not hold its usual meaning, for example; if malathion of 80% purity is dissolved in various amounts of acetone, to get solutions of 20, 30 or 40%, and then applied topically, the given concentration will be held for a few seconds, and finally only 80% of the malathion will be left in every case. In other words, no concentration difference will be obtained. However, there is a difference in terms of the residue left on the cuticle. Let us assume that 0.1 gamma of malathion in a given area of 0.07 mm^2 is composed of 5 particles (in this case molecules). Then 10 molecules will be present in 0.2 gamma of malathion. This gives a concentration in terms of amount per unit area or dosage and the relation will be held until the whole area is completely covered by malathion molecules. Therefore, in the strict sense, the permeability constant given in <u>topical applications</u> cannot be compared with the permeability constant obtained from the isolated cuticle.

In a previous chapter (3.4.1.) a theoretical diffusion equation is obtained. However a great simplification

was necessary to obtain a simple equation for penetration. Thus the penetration equation (Eq.16) describes the actual penetration within a limited range. The significance of Eq.16 will be discussed here.

In the process of obtaining the penetration equation the differential equation was not actually solved:

$$\partial c/\partial t = a^2 \cdot \nabla^2 c \qquad (9)$$

was assumed to be

$$dc/dt = k(C_o - C_i) \qquad (11)$$

under the assumption that the initial concentration is very high, i.e. $(C_o - C_i)$ is constant throughout the reaction, but this relation is not held after a certain time of reaction.

To test the applicability of the equation the differential equation (9) will have to be solved.

Let us assume the dimensionless concentration as follows:

$$\sigma = (C - C_s)/(C_o - C_s)$$

Where C is the concentration at any point, C_s the equilibrium concentration, and C_o is the initial concentration of the system. For $C = C_o$, we get $\sigma = 1$, and on the side and upper surfaces, where $C - C_s$, we have $\sigma = 0$. Since the derivatives of C and σ are same except for a multiplicative constant, the boundary condition at $x = 0$ will be $\partial\sigma/\partial x = 0$, so that the conditions on σ are homogeneous. The diffusion equation is:

$$\partial\sigma/\partial t = k\nabla^2\sigma \qquad (25)$$

Now the condition is; (1) σ is finite throughout the volume at all times, and (2) $\partial\sigma/ x = 0$ at $x = 0$. In cylindrical co-ordinates, the equation becomes

$$(\partial\sigma/\partial t) = k\left((1/r)\frac{\partial}{\partial r}\left(r\cdot\frac{\partial\sigma}{\partial r}\right) + (1/r^2)\frac{\partial^2\sigma}{\partial\theta^2} - \frac{\partial^2\sigma}{\partial x^2}\right) \qquad (26)$$

The required solution will be independent of θ

Let assume:

$$\sigma = R(r)X(x)T(t) \qquad (27)$$

Putting this in (26) we obtain:

$$\frac{1}{T}\cdot\frac{\partial T}{\partial t} = k\frac{1}{rR}\cdot\frac{d}{dr}\left(r\frac{dR}{dr}\right) + k.\frac{1}{x}\cdot\frac{d^2X}{dx^2} \qquad (28)$$

This can be satisfied if we make the assumption that:

$$(1/rR)\frac{d}{dr}\left(r\frac{dR}{dr}\right) = -\alpha^2 \qquad (29)$$

$$\frac{1}{x}\frac{d^2X}{dx^2} = -\beta^2 \qquad (30)$$

$$r^2(d^2R/dr^2) + r(dR/dr/ + \alpha^2 r^2 R = 0$$

$$\left\{ \begin{array}{ll} d^2X/dx^2 + \beta^2 X = 0 & (32) \\ dT/dt + k(\alpha^2 - \beta^2)\,T = 0 & (33) \end{array} \right.$$

The solutions of these equations are

$$R = C_1 J_0(\alpha r) + C_2 Y_0(\alpha r) \tag{34}$$

$$X = C_3 \cos\beta x + C_4 \sin\beta x \tag{35}$$

$$T = C_5 e^{-k(\alpha^2 + \beta^2)t} \tag{36}$$

The product solution of Eq.(27) will be of the form:

$$\sigma = C_5(C_1 J_0(\alpha r) + C_2 Y_0(\alpha r))(C_3 \cos\beta x + C_4 \sin\beta x) e^{-k(\alpha^2+\beta^2)t} \tag{37}$$

The solution can be interpreted as follows; since σ was defined as:

$$\sigma = \frac{C - Cs}{Co - Cs} \tag{38}$$

$$C = \sigma Co + Cs(1 - \sigma) \tag{39}$$

Moreover C can be expressed in terms of C : since at equilibrium the concentration of solution in the diffusing cell (V_i in volume) must be the same as that in the diffusion cell (V in volume),

$$Cs = S_i/V_i = (S_o - S_i)/V \qquad (40)$$

where S_i is the amount of the material dissolved with the solvent in the diffusing cell, and S_o is that of in the diffusion cell. Thus

$$C_s = C_o(V/V - V_i)) \qquad (41)$$

Now the change of concentration at a geographic point $P(r1 \,.\, x_1)$ is

$$C = C_o\Big(C_5\big(C_1 J_o(\alpha r_1) + C_2 Y_o(\alpha r_1)\big)\big(C_3 \cos\beta x_1 + C_4 \sin\beta x_1\big) e^{-k(\alpha^2-\beta^2)t}$$
$$+ C_s\Big(1 - C_5\big(C_1 J_o(\alpha r_1) - C_2 Y_o(\alpha r_1)\big)\big(C_3 \cos\beta x_1 - C_4 \sin\beta x_1\big) e^{-k(\alpha^2+\beta^2)t}$$

(42)

Since r_1 and x_1 are certain value, we can rewrite the equation:

$$C_5[C_1 J_o(\alpha r_1) + C_2 Y_o(\alpha r_1)][C_3 \cos\beta x_1 + C_4 \sin\beta x_1) = -Q \qquad (43)$$

Here Q is a certain constant. Then Eq.(42) will be:

$$C = -C_o Q e^{-k(\alpha^2-\beta^2)t} + C_s(1 - Q e^{-k(\alpha^2+\beta^2)t} \qquad (44)$$

In addition the equation can be simplified by considering Eq. (41) as follows:

$$C - C_o\left((V/(V+V_i)) - \frac{V_i}{V+V_i} Q e^{-k(\alpha^2+\beta^2)t}\right) \qquad (45)$$

The solution can be summarized into the following equation:

$$C = Co \frac{V}{V+V_i}\left(1 - \frac{Vi}{V} Q e^{-k(\alpha^2+\beta^2)t}\right) \qquad (46)$$

Now let us compare the Eq. (46) with the original penetration equation Eq. (16), on which the calculation of the permeability constant is based. The concentration varies proportionally with the original concentration and exponentially with time; thus the original assumption is satisfied.

5. APPENDIX

-Determination of the Permeability Constant in the Isolated cuticle.

As a result of previous considerations (chapter 4), the permeability constant of the cuticle of P. americana was obtained in this experiment. The pronotum was carefully separated from roaches with the intact epicuticle, endocuticle, and epidermis, and attached to the diffusing tube, which has been illustrated in Fig. 1. The tube with the cuticle was kept for 1 day in a container of which the humidity was maintained at 80% R.H. by concentrated sulfuric acid. After this treatment the tube was set in a diffusion cell (Richards and Fan, 1949, Fig.1). Radioactive malathion was poured into the diffusing cell. After one hour (see also 2.2.2.2.)the radioactivity in the diffusion cell, in the cuticle, and in the diffusing tube were measured. The results are shown in Table 27.

Now let us calculate the diffusible intact volume by comparing the value with the permeability constant which was obtained from the intact, dead insect by means of topical application.

Table 27. Calculated permeability constant by using the isolated cuticle

Counts in the diffusing tube	Counts in the diffusion cell	Counts in the cuticle	Permeability const.
c/3min.	c/3min.	c/3min.	cm/sec.
1671	9	70	7.074×10^{-7}
5471	17	100	4.892×10^{-7}

Temperature 22.7'C
45% R.H.
Penetration for 1 hour.

The diffusible intact volume may be defined as follows: it is the volume of actual body part which is utilized for diffusion. Therefore the permeability constant in the isolated cuticle, P is obtained for Eq. (16) as:

$$P_i = \frac{\text{the diffusible intact volume}}{A \cdot t} \ln \frac{Co}{Co - Ci} \qquad (47)$$

Whereas the permeability constant in the intact cuticle P_{in} was obtained as follows:

$$P_{in} = \frac{\text{Volume of the insect}}{A \cdot t} \ln \frac{Co}{Co - Ci} \qquad (48)$$

Therefore the ratio x is:

$$\frac{\text{the diffusible intact volume}}{\text{volume of the insect}} = \frac{P_i}{P_{in}} \qquad (49)$$

$$= x$$

According to Table 19, the value of the permeability constant for one hour is 2.975×10^{-5}, whereas the average permeability constant in Table 27 is 5.98×10^{-7}. Accordingly:

$$x = \frac{Pi}{Pin}$$

$$= \frac{5.98 \times 10^{-7}}{2.975 \times 10^{-5}}$$

$$= 2.01 \times 10^{-2}$$

That is:

<u>The diffusible intact volume</u>

= the volume of the insect $X(2 \times 10^{-2})$

Here it can be concluded that the actual volume which is utilized as a diffusion cell in <u>the topical application</u> is only 1/50 of the insect body.

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